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PRINCIPAL INVESTIGATOR: Serdar E. Bulun, M.D.

CONTRACTING ORGANIZATION: The University of Illinois at Chicago
Chicago, IL 60612-7227

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Serdar E. Bulun, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Illinois at Chicago
Chicago, IL 60612-7227

E-Mail: PI - sbulun@uic.edu
Office of Research Services - awards@uic.edu

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13. ABSTRACT ***(Maximum 200 Words)***

We hypothesize that breast malignant epithelial cells interact with surrounding stroma to provide biochemical and structural support for tumor tissue. Specifically, malignant epithelial cells block the differentiation of surrounding adipose fibroblasts through cytokines. Additional epithelial factors strikingly induce aromatase expression in these undifferentiated fibroblasts via switching aromatase gene promoter use from the physiologically used promoter I.4 to aberrantly activated promoter II. During this entire grant period, we have accomplished all specific aims. First, we showed that factors secreted by malignant epithelium inhibit differntiation of adipose fibroblasts to mature adipocytes. These epithelial factors were identified as TNF and IL-11. TNF and IL-11 suppress essential adipogenic transcription factors C/EBPα and PPARγ in undifferentiated adipose fibroblasts. Since aromatase expression resides only in undifferentiated fibroblasts but not in mature adipocytes, inhibition of differentiation serves to increase aromatase-expressing cells around malignant epithelial cells. As a second hit, malignant epithelium secretes additional factors other than TNF or IL-11 to upregulate aromatase expression in adipose fibroblasts. We demonstrated that the transcription factor C/EBPβ mediates this malignant epithelial cell effect on adipose fibroblasts. C/EBPβ binds to promoter II to activate this pathologic promoter in fibroblasts in tumor tissue. The end result is aromatase overexpression and increased local formation of estrogen in breast cancer.

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5. INTRODUCTION

This Final Report was prepared as a requirement of the Idea Award entitled "Adipocyte Differentiation: Relationship to Breast Cancer" funded by the US Army Medical Research and Materiel Command Breast Cancer Research Program (DAMD17-97-1-7025). This report covers research for the entire period from 10/1/1997 until 3/22/2002. We will initially provide an overview of this research work. Then, details will be provided in **6. BODY**. The long term objective of this project is to characterize the cellular and molecular mechanisms responsible for intra- and peritumoral accumulation of stromal fibroblasts. To achieve this objective, the following specific aims were proposed. The first **specific aim 1 is *to determine whether secretory products of breast cancer cells prevent differentiation of adipose fibroblasts into mature adipocytes***. Major goals in this aim have been accomplished and detailed in **6. BODY**. Briefly, malignant epithelial but not benign epithelial cells secrete factors that inhibit adipocyte differentiation. **Specific Aim 2 is *to characterize the secretory products of malignant epithelial cells, which downregulate adipogenic transcription factors***. This aim has also been accomplished completely and results were detailed in **6. BODY**. Briefly, these malignant cell-derived anti-adipogenic factors were identified as TNF and IL-11. TNF and IL-11 accomplish inhibition of differentiation by specifically suppressing essential adipogenic factors, namely C/EBPα and PPARγ, in adipose fibroblasts. **Specific Aim 3 is *to determine whether adipogenic transcription factors regulate aromatase P450 expression in human adipose fibroblasts***. This aim has also been accomplished completely and results were detailed in **6. BODY**. Briefly, malignant epithelial cells upregulate C/EBPβ in the surrounding undifferentiated fibroblasts. C/EBPβ *per se* is incapable of differentiating fibroblasts but binds to aromatase promoter II and increases estrogen production in these undifferentiated cells. **Specific Aim 4 is *to determine the regional distribution of C/EBPβ, PPARγ, C/EBPα and P450arom expression in the breast in relation to tumor location.*** Finally, aim 4 was also accomplished completely and results were detailed in **6. BODY**. Briefly, we found that the essential adipogenic transcription factor C/EBPα was suppressed, whereas the aromatase-stimulating transcription factor C/EBPβ and aromatase are upregulated in undifferentiated fibroblasts adjacent to malignant epithelial cells. In adipose tissue distant to epithelial cells, however, these effects disappear.

6. BODY

Our findings in relation to each aim are detailed below:

Aims 1 and 2: Malignant breast epithelial cells induce a reaction in the adjacent adipose stroma characterized by accumulation of large numbers of fibroblasts, *i.e.*, desmoplastic reaction. This dense layer of peritumoral fibroblasts arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. Here we seek to determine the epithelial-stromal interactions responsible for desmoplastic reaction using 3T3-L1 murine fibroblasts and human adipose fibroblasts, which differentiate to mature adipocytes as model systems. The following is a more detailed description of our findings regarding aims 1 and 2:

After exposure to an activating cocktail of hormones for two days, control 3T3-L1 cells differentiated fully to mature adipocytes by days 6-8. Coculturing 3T3-L1 cells with T47D or MCF7 breast cancer cell lines inhibited this differentiation almost completely. Likewise, T47D-cell-conditioned medium gave rise to inhibition of the differentiation of 3T3-L1 cells. T47D-cell-conditioned medium also inhibited the differentiation of human breast adipose fibroblasts in primary culture, whereas control cells differentiated to mature adipocytes. This tumor effect was eliminated using neutralizing antibodies against TNF or IL-11. **(APPENDIX 1)**. TNF mRNA was demonstrated by northern analysis in T47D cells treated with lipopolysaccharide or TNF itself but not in 3T3-L1 cells indicating that T47D cells represent the major source of TNFα production in this co-culture system **(APPENDIX 1).** Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors including C/EBPδ or C/EBPβ, PPARγ and C/EBPα. We demonstrated by northern analysis that exposure of 3T3-L1 cells to T47D-cell-conditioned medium upregulated C/EBPδ or CEBPβ expression but suppressed or inhibited the expression of PPARγ and C/EPBα in 3T3-L1 cells treated with the cocktail **(APPENDIX 1).** In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of aP2 mRNA, which is an adipocyte-specific gene **(APPENDIX 1).** Treatment of 3T3-L1 cells with T47D cell-conditioned medium or TNF changed neither the numbers of cells in G_O/G_1 or S phases nor the rate of [^{3}H]thymidine incorporation into these cells, thus, ruling out a proliferative effect of malignant cells on the surrounding fibroblasts **(APPENDIX 1)**. In summary desmoplastic reaction primarily occurs via the action of cytokines (TNF and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the inhibition of PPARγ and C/EBPα expression.

Aim 3: Expression of aromatase P450 (P450arom), which catalyzes the formation of estrogens, is aberrantly increased in adipose tissue fibroblasts surrounding breast carcinomas giving rise to proliferation of malignant cells. Aromatase in human adipose tissue is expressed primarily in undifferentiated fibroblasts under the control of distinct and alternatively used P450arom promoters. In tumor-free breast adipose tissue, P450arom is expressed at low levels via a distal promoter (I.4), whereas in the breast

adipose tissue bearing a tumor, P450arom is increased through the activation of two proximal promoters, II and I.3. Since the *in vivo* activation of P450arom promoter II is a key event responsible for aberrantly high P450arom expression in breast tumors, we studied the molecular basis for the enhancement of P450arom promoter II using human adipose tissue fibroblasts (HAF) in primary culture treated with T47D or breast cancer cell-conditioned medium (TCM) as a model system. These findings are detailed below:

Upon treatment by TCM, HAF displayed a striking induction of P450arom mRNA levels via promoter II usage **(APPENDIX 2)**. This effect appeared to be specific for malignant breast epithelial cells, because conditioned media from breast cancer cell lines T47D and MCF-7 induced promoter II activity, whereas normal breast epithelial cells or liver or prostate cancer cell lines did not produce such an effect. Although treatment with a cAMP analog also caused a switch in the promoter use from I.4 to II in cultured HAF, TCM-induced promoter II use was found to be mediated via a cAMP-independent pathway **(APPENDIX 2)**. Use of serial deletion mutants of the promoter II 5′-flanking sequence revealed the presence of critical *cis*-acting elements within the –517/-278 bp region, which regulate the baseline and TCM-induced activities **(APPENDIX 2)**. TCM caused a 5.7-fold induction of the –517 bp/promoter II construct, whereas site directed mutagenesis of a CCAAT/enhancer binding protein (C/EBP) binding site (-317/-304 bp) abolished both baseline and TCM-induced activities **(APPENDIX 2)**. Ectopic expressions of C/EBPβ and to a lesser extent C/EBPα, but not C/EBP, induced promoter II activity **(APPENDIX 2)**. Effects of TCM and C/EBPβ were not found to be additive on promoter II activity **(APPENDIX 2)**. Moreover, we demonstrated the presence of both C/EBPβ and C/EBPα but not C/EBPα in a DNA-protein complex formed by the nuclear extract from TCM-treated HAF and a probe containing the critical C/EBP binding element (-317/-304 bp) **(APPENDIX 2)**. Finally, treatment of HAF with TCM strikingly induced C/EBPβ mRNA levels, whereas this did not affect the levels of C/EBPα or C/EBPδ mRNA, as determined by northern analysis **(APPENDIX 2)**. In conclusion, malignant breast epithelial cells secrete factors, which induce P450arom expression in adipose tissue fibroblasts via promoter II. This is, at least in part, mediated by malignant epithelial cell-induced upregulation of C/EBPβ and enhanced binding of this transcription factor to a critical regulatory element (–317/-304 bp) upstream of promoter II.

Aim 4. Immunohistochemistry for the in vivo distribution of significant molecules identified in this grant:

During this grant period, we employed immunohistochemistry to determine the distribution of C/EBPα, C/EBPβ and C/EBPγ in human mastectomy specimens. These results confirmed our findings and hypothesis summarized above: We used 30 biopsies from 10 mastectomy specimens removed for breast cancer. We determined the expression of C/EBPα, C/EBβ and C/EPBδ in fibroblasts within the tumor sample (intratumoral), within adipose tissue biopsied 1 cm from the tumor (adjacent) and within adipose tissue biopsied 4 cm from the tumor (distant). **APPENDIX I** illustrates fibroblasts and adipocytes with immunoreactive nuclei for these transcription factors. C/EBPα was not detectable in intratumoral fibroblasts, but it was readily detectable in fibroblasts and adipocytes in adjacent and distant fat tissue biopsies from 10 patients **(APPENDIX I)**. No differences were observed in the distribution of expression of C/EBβ and C/EPBδ. An H-scoring system was used to determine the

number of immunoreactive fibroblasts, as described in Materials and Methods **(APPENDIX I)**. This illustrated strikingly suppressed expression of C/EBPα in intratumoral fibroblasts. These results are in agreement with those of the northern analysis **(APPENDIX I)**.

Previous experiments have suggested that IL-11 and TNF **(APPENDIX I)** mediated the inhibition of adipocyte differentiation by cancer cells. Thus, during this report period, we determined the cellular origin of these cytokines in the breast cancer. First, we demonstrated TNF transcripts in stimulated T47D breast cancer cells but not in 3T3-L1 cells employing northern blotting. **(APPENDIX I)**. A full-length murine TNF cDNA was used to probe the membrane. Next, we determined the *in vivo* cellular distribution of immunoreactive IL-11 and TNFα in 15 mastectomy specimens **(APPENDIX I)**. All malignant epithelial cells showed intense cytoplasmic staining for these two cytokines, whereas less than 25% of fibroblasts were immunoreactive with considerably less staining intensity **(APPENDIX I).**

7. KEY RESEARCH ACCOMPLISHMENTS

- We report hereby complex epithelial-stromal interactions between malignant breast epithelial cells and surrounding adipose fibroblasts (preadipocytes) to develop a model whereby malignant cells maximize the number of surrounding undifferentiated adipose fibroblasts and stimulate estrogen production in these fibroblasts.

- Malignant breast epithelial cells inhibit adipocyte differentiation specifically via the suppression of the essential adipogenic transcription factor C/EBPα but not C/EBPβ or C/EBPδ, as demonstrated by the detection of these nuclear factors in sections of human breast tumors C/EBPβ and C/EBPδ are not essential or sufficient by themselves for adipocyte differentiation.

- Malignant epithelial cells are the major sites of expression of IL-11 and TNFα, which inhibit adipocyte differentiation.

- Malignant breast epithelial cells stimulate the expression of C/EBPβ in adjacent adipose tissue fibroblasts.

- Binding of C/EBPβ to aromatase promoter II activates this promoter and switches the promoter use from the physiologically used promoter I.4 to II in adipose fibroblasts treated with cancer cell-conditioned medium. This ultimately gives rise to a striking increase in total aromatase mRNA levels. Our previous in vivo data also confirm that promoter II is preferentially activated in adipose tissue adjacent to breast tumors. Thus, our current findings provided a molecular basis for this observation.

- These mechanistic in vitro findings are supported by the cell-specific distribution of cytokines and transcription factors in breast tumor biopsies.

8. REPORTABLE OUTCOMES

The following manuscripts bear the acknowledgement of this grant:

1. Meng L, J Zhou, H Sasano, T Suzuki, K Zeitoun, and **SE Bulun**: TNFα and IL-11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively downregulating C/EBPα and PPARγ: mechanism of desmoplastic reaction. *Cancer Research.* 61:2250-2255 (2001).
2. Zhou J, B Gurates, S Yang, S Sebastian and **SE Bulun**: Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by C/EBPβ. *Cancer Research.*61:2328-2334 (2001).

9. CONCLUSIONS

Our results during this grant period supported the original central hypothesis. In summary, we have a two-hit hypothesis. First, malignant epithelial cells block the differentiation of surrounding adipose fibroblasts through cytokines TNF and IL-11. Then, yet unidentified epithelial factors other than these cytokines induce aromatase expression in these undifferentiated fibroblasts via switching aromatase gene promoter use from the physiologically used promoter I.4 to aberrantly activated promoter II. We demonstrated that the transcription factor C/EBPβ mediates this malignant epithelial cell effect on adipose fibroblasts. The end result is aromatase overexpression and increased local formation of estrogen in breast cancer. In future, we seek to isolate these malignant epithelial cell-derived factors and associated signaling pathways in adipose fibroblasts.

10. REFERENCES

Please refer to references provided in the APPENDIX.

11. APPENDIX

Two Cancer Research publications that bear this grant number were attached.

[CANCER RESEARCH 61, 2250–2255, March 1, 2001]

Tumor Necrosis Factor α and Interleukin 11 Secreted by Malignant Breast Epithelial Cells Inhibit Adipocyte Differentiation by Selectively Down-Regulating CCAAT/Enhancer Binding Protein α and Peroxisome Proliferator-activated Receptor γ: Mechanism of Desmoplastic Reaction[1]

Li Meng,[2] Jianfeng Zhou,[2] Hironobu Sasano, Takashi Suzuki, Khaled M. Zeitoun, and Serdar E. Bulun[3]

Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235 [L. M., K. M. Z.]; Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60612-7313 [J. Z., S. E. B.]; and Department of Pathology, Tohoku University School of Medicine, Sendai 980, Japan [H. S., T. S.]

ABSTRACT

The dense layer of fibroblasts that accumulate around malignant breast epithelial cells (*i.e.*, desmoplastic reaction) arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. We report herein a number of epithelial-stromal interactions responsible for desmoplastic reaction in breast cancer using cultured 3T3-L1 murine fibroblasts and human adipose fibroblasts, which can be activated with a mixture of hormones to differentiate to mature adipocytes. Adipocyte differentiation was inhibited by coculturing fibroblasts with various breast cancer cell lines (T47D, MCF-7, SSC202, SSC78, and SSC30) completely or by breast cancer cell conditioned media in a dose-dependent manner; on the other hand, adipocyte differentiation was not inhibited by coculturing with normal human primary mammary epithelial cell conditioned medium. This tumor effect was eliminated using neutralizing antibodies against tumor necrosis factor (TNF)-α or interleukin (IL)-11. TNF-α and IL-11 levels were 2.5–3 times higher in T47D conditioned medium compared with control medium, and TNF-α transcripts were detectable in T47D but not in 3T3-L1 cells in culture, indicating that the malignant epithelial cell is the major site of cytokine production. This was confirmed *in vivo* in mastectomy specimens, where immunoreactive TNF-α and IL-11 were readily detectable in malignant epithelial cells but not in the majority of the surrounding fibroblasts. Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors, including CCAAT/enhancer binding protein (C/EBP)β, C/EBPδ, peroxisome proliferator-activated receptor (PPAR)γ and C/EBPα. C/EBPα and PPARγ are essential for this process. We demonstrated by Northern analysis that exposure of activated 3T3-L1 cells to T47D cell conditioned medium strikingly decreased the levels of PPARγ and C/EBPα transcripts and increased the levels of C/EBPβ and C/EBPδ transcripts. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of *aP2* mRNA, which is an adipocyte-specific gene. These *in vitro* observations were confirmed in sections of human malignant breast tumors, where immunoreactive C/EBPα was readily detectable in adipose fibroblasts distant to the tumor but not in intratumoral fibroblasts. Treatment of 3T3-L1 cells with T47D cell conditioned medium or TNF-α changed neither the numbers of cells in G_0-G_1, S, and G_2 phases nor the rate of [^{3}H]thymidine incorporation, thus ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary, desmoplastic reaction primarily occurs via the action of cytokines (TNF-α and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of expression of the essential adipogenic transcription factors, *i.e.*, PPARγ and C/EBPα.

INTRODUCTION

Breast tumors are characterized by the accumulation of fibroblasts adjacent to malignant epithelial cells, which is commonly known as the desmoplastic reaction (1). In fact, the majority of breast cancers have been referred to as "scirrhous" because of their extremely hard consistency provided by large numbers of fibroblasts dispersed between malignant epithelial cells, as well as within the immediate periphery of tumors. The relationship between adipose stroma and breast cancer is unique in the sense that stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells greatly influence the composition of the adjacent tissue. Evidence from several laboratories indicates that this epithelial-stromal interaction also involves paracrine mechanisms that promote the development and growth of breast carcinomas (2–4). These morphologically identified intra- and peritumoral fibroblasts originate from adipose tissue and most likely represent potential preadipocytes, because fibroblasts isolated from adipose tissue are capable of differentiating to mature adipocytes under defined culture conditions (5, 6). We hypothesized that malignant epithelial cells of breast tumors secrete growth factors and cytokines to prevent the differentiation of fibroblasts to mature adipocytes in the adjacent adipose tissue. The following body of preliminary data supported this hypothesis:

(*a*) Tumors were found in breast quadrants with the highest P450arom[4] transcript levels and the highest fibroblast:adipocyte ratio (7). This parallelism between the distribution of fibroblasts and aromatase expression is not surprising, because aromatase is a marker for undifferentiated adipose fibroblasts (8, 9).

(*b*) In the cancer-free human breast, the highest fibroblast:adipocyte ratios and P450arom transcript levels were found in the lateral and upper region (10). This distribution roughly correlates with the most common sites where infiltrating duct carcinomas develop.

(*c*) Breast tumor conditioned medium was found to induce aromatase activity in adipose fibroblasts (11). This tumor-induced effect can be inhibited and titrated by addition of an anti-IL-11 antibody.[5] When aromatase expression is viewed as a fibroblast marker, these results suggested that malignant epithelial cells secrete factors, such as IL-11, to prevent differentiation of fibroblasts. Additionally, estradiol

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[2] These authors contributed to this work equally.

[3] To whom requests for reprints should be addressed, at Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, 820 South Wood Street, M/C 808, Chicago, IL 60612. Phone: (312) 996-8197; Fax: (312) 996-4238; E-mail: sbulun@uic.edu.

[4] The abbreviations used are: P450arom, aromatase P450; IL, interleukin; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; DEX, dexamethasone; MIX, 1-methyl-3-isobutyl-xanthine; TCM, T47D cell conditioned medium; NCM, normal mammary epithelial cell conditioned medium.

[5] J. E. Nichols, S. E. Bulun, and E. R. Simpson, unpublished observations.

pretreatment of cancer cells potentiated aromatase induction in a dose-dependent fashion, which is suggestive of a paracrine loop (11). Moreover, we demonstrated that certain adipogenesis inhibitors such as IL-11 are secreted by the T47D breast cancer cell line (12). Again, estradiol stimulated IL-11 expression in T47D cells in a dose-dependent fashion (12).

(*d*). IL-11 and TNFα stimulation of the fibroblast marker aromatase in adipose fibroblasts can be interpreted as inhibition of adipocyte differentiation, because aromatase expression in adipose tissue primarily resides in fibroblasts but not in mature adipocytes (8, 9, 13, 14).

There may be multiple potential mechanisms responsible for accumulation of adipose fibroblasts within the tumor and in adjacent stroma. It is possible that fibroblasts proliferate in response to tumor-derived growth factors. Although treatment of murine 3T3-L1 fibroblasts in culture with serum, insulin, insulin-like growth factor I and epidermal growth factor initially causes proliferation, cell replication rapidly stops under these conditions, and fibroblasts eventually differentiate into mature adipocytes (15–18). Thus, it follows that there have to be other effective mechanisms for peritumoral fibroblast accumulation. Inhibition of differentiation or dedifferentiation of preexisting adipocytes may provide these critical mechanisms responsible for extremely high fibroblast:adipocyte ratios in the stroma surrounding cancer cells. This process may be under the control of cytokines secreted by the malignant epithelial cells.

The cellular and molecular mechanisms responsible for the differentiation of stromal fibroblasts into mature adipocytes have been well characterized (19–24). During mammalian development, embryonic mesoderm gives rise to several highly specialized cell types, including adipocytes. Differentiation of adipocytes from multipotential fibroblastic precursors is primarily controlled by two tissue-specific transcription factors: the C/EBPα and PPARγ. During the process of differentiation, C/EBPβ (and possibly C/EBPδ) are initially expressed and convert multipotential mesenchymal precursor cells into preadipocytes (19). These "determined" preadipocytes are able to respond subsequently to potent adipogenic inducers such as PPARγ. The PPAR isoform PPARγ (21) is a member of the ligand-activated transcription factor family that heterodimerizes with retinoid X receptor α and binds to the promoters of adipocyte-specific genes. A third adipocyte-enriched transcription factor, C/EBPα, has been shown to promote terminal adipocyte differentiation (20). When expressed together, PPARγ and C/EBPα act synergistically to powerfully promote adipocyte differentiation in fibroblastic cells, regardless of tissue of origin (22, 24). For practical purposes, we will refer to undifferentiated mesenchymal precursors and determined preadipocytes in human adipose tissue as adipose fibroblasts, because both cell types appear as fibroblasts morphologically. Fibroblasts isolated from adipose tissue differentiate into adipocytes when cultured in a defined medium (5, 6). On the other hand, certain substances, such as TNF-α, are not only capable of inhibiting adipocyte differentiation but also of reversing it by suppressing the expression of PPARγ (25, 26). Most of the work in this field has been performed using rodent fibroblasts and has been related to obesity and diabetes. Possible roles of malignant epithelial cells in paracrine regulation of these transcription factors and on adipocyte differentiation have not been studied to date. We report herein a number of epithelial-stromal interactions in the breast cancer, which represent cellular and molecular mechanisms responsible for the development and maintenance of desmoplastic reaction.

MATERIALS AND METHODS

Tissue Acquisition. Breast adipose tissue was obtained from five patients undergoing reduction mammoplasty. These tissues were immediately processed for primary cultures of adipose fibroblasts. Breast cancer and surrounding adipose tissue samples were obtained from 25 mastectomy specimens for immunohistological detection of C/EBPs (α, β, and δ), IL-11, and TNF-α. These studies were conducted following protocols approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Tohoku University School of Medicine.

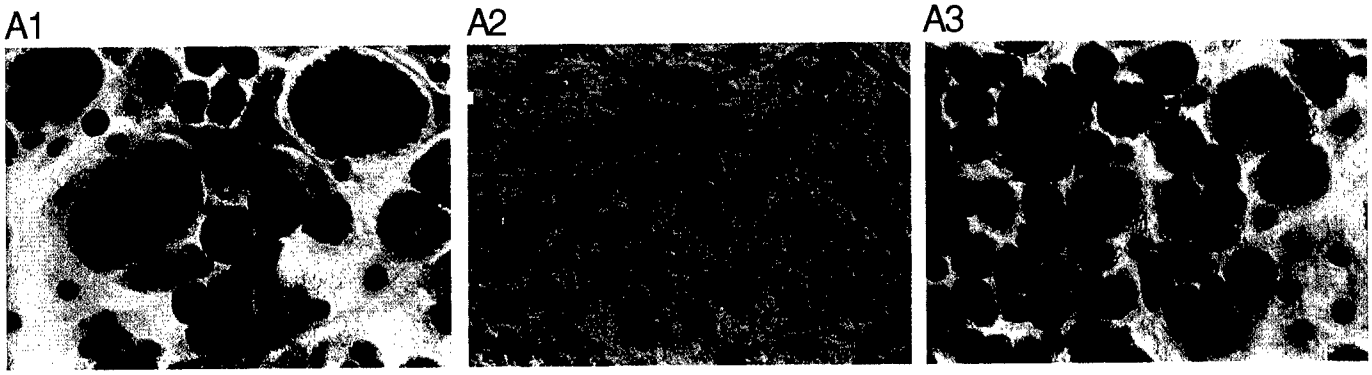
Detection of Transcripts of Adipocyte-specific Genes and Cytokines by RNA Blot Analysis. Total RNA was isolated from fibroblasts/adipocytes in culture, electrophoretically fractionated (10 μg), and transferred to a charged membrane. Duplicate measurements of absorbance (260 μm) were performed to equalize loading, which was confirmed by visual inspection of 18S and 28S RNA stained with ethidium bromide. Northern blots were hybridized with cDNA probes labeled by random priming using [^{32}P]dCTP. cDNA templates for adipocyte P2 (aP2), C/EBPδ, C/EBPβ, PPARγ, C/EBPα, and TNF-α were kindly provided by Drs. Steve McKnight, Gokhan Hotamisligil, Carol Mendelson, Bruce Beutler, and Gretchen Darlington.

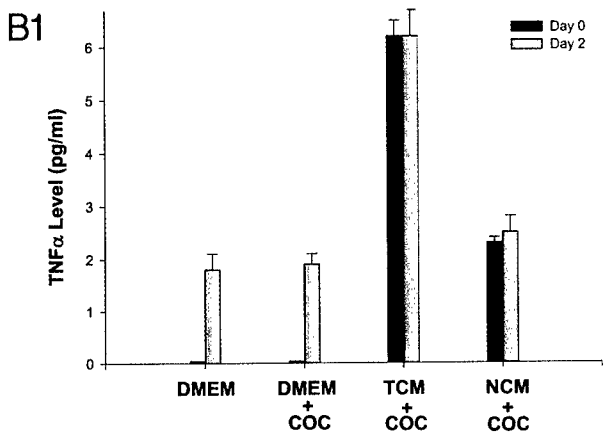
Cell Cultures and Differentiation of Fibroblasts to Adipocytes. We routinely performed primary cultures of human adipose fibroblasts as described previously (8). Differentiation of human adipose fibroblasts to mature adipocytes was performed following a modified protocol originally outlined by Hauner *et al.* (5). Breast adipose tissue, obtained from women at the time of reduction mammoplasty, was processed by mincing, washing, digestion (with collagenase), and centrifugation steps. The floating mature adipocytes were aspirated, and the sedimented fibroblast fraction was resuspended in DMEM with 10% FCS as described previously (8). Nucleus-containing cells were inoculated at a density of 50,000/cm^2 into six-well plates. Cultures were grown for a 24-h period in DMEM with 10% FCS. Cells were then placed in a chemically defined phenol red-free and serum-free medium consisting of DMEM/F12 (1:1, v/v), 15 mM $NaHCO_3$, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 0.67 μM human insulin, 0.2 mM triiodothyronine, 0.5 mM DEX, and antibiotics for 21 days. Within 15–21 days, cells achieve maximum differentiation. Cells were regarded as differentiated by morphological criteria when, after acquiring a round shape, the cytoplasm was completely filled with multiple lipid droplets as assessed by Oil Red O staining. The proportion of differentiated cells is estimated by direct counting under the microscope of total and differentiated cells, using a micrometer.

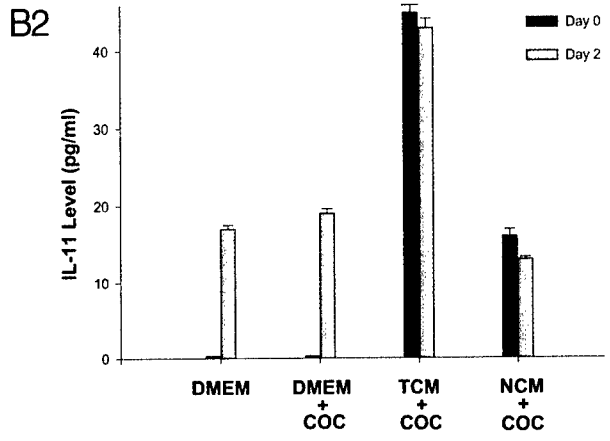
The 3T3-L1 fibroblasts were grown in DMEM with 10% FCS. T47D cells were initially grown in RPMI 1640 with 10% FCS containing 0.02 mM HEPES, whereas MCF-7 cells were grown in MEM with 10% FCS until confluent. SSC202, SC78, and SSC30 breast cancer cells (kindly provided by Dr. Adi Gazdar) were grown to confluence in DMEM with 10% FCS. Primary human mammary epithelial cells purchased from Clonetics, Inc. (Walkersville, MD) were grown to confluence in the MEGM medium supplied by the manufacturer (Clonetics). Cell conditioned medium from T47D and human mammary epithelial cells were collected for coculture experiments in the following fashion. Confluent cells were maintained in DMEM for 12 h for washout. Then, cells were incubated in DMEM for 24 h to allow accumulation of factors secreted by these cells. These conditioned media were subsequently used for coculture experiments. All cells were incubated at 37°C in 5% CO_2. To induce the adipogenic differentiation of 3T3-L1 fibroblasts within 2 days of reaching confluence, these cells were treated with DEX (0.25 μm), MIX (0.5 mM), and insulin (1 μg/ml) for 2 days and then maintained in DMEM with 10% FCS for 6 additional days. Cells containing multiple fat droplets were scored as differentiated by phase contrast microscope after staining with Oil Red O. All culture media were phenol red free.

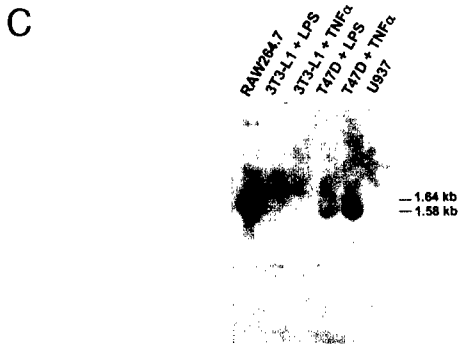
Neutralizing antibodies against human IL-11 (Ab-218-NA), TNF-α (Ab-210-NA), IL-2, and normal goat IgG (AB-108-C) were purchased from R&D Systems, Inc. (Minneapolis, MN). These were added to the conditioned medium at a final concentration of 40 ng/ml.

As an alternative to the use of breast cancer cell conditioned media, 3T3-L1 and human adipose fibroblasts were cocultured with breast cancer cells using 35-mm, six-well plates. 3T3-L1 cells or adipose fibroblasts were plated on the bottom wells. Breast cancer cells were seeded on the permeable membrane (0.45-μm) tissue culture inserts. 3T3-L1 cells were cultured in DMEM with 10% FCS, after they reached confluence for 2 days. At this time, inserts containing cancer cells were introduced, and the medium was changed to the appropriate differentiation medium for 48 h. Then the medium was switched back to DMEM plus 10% FCS for 6 days. In the case of human adipose fibroblasts, cocultures were maintained for 15–20 days in the differentiating









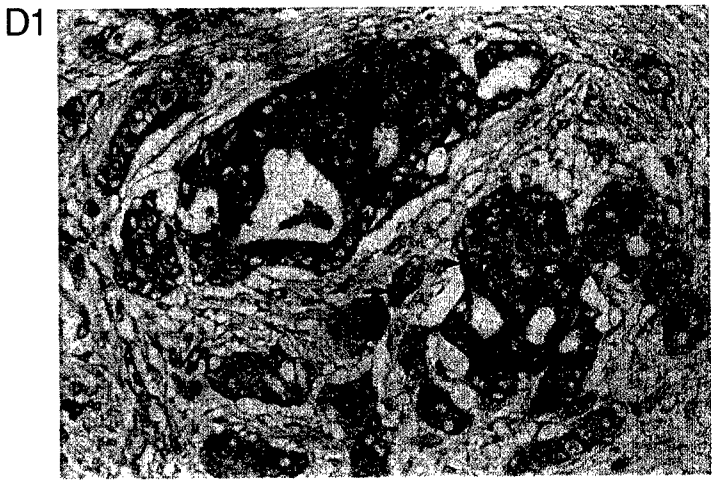


Fig. 1. 3T3-L1 cells, 8 days after reaching confluence. Confluent cells were treated with the differentiating cocktail (DEX, insulin, and MIX) for the first 2 days and then were maintained in DMEM for the next 6 days. Accumulation of lipid droplets represented by the *red* stain was detected in the cytoplasm of 80% of adipocytes (*A1*; stain, Oil Red O). Adding medium conditioned with breast cancer cells (in this instance, T47D cells) completely inhibited their differentiation to adipocytes, evident by the lack of lipid droplets (*A2*). After neutralizing antibodies against IL-11 and TNF-α at the final concentrations of 40 μg/ml were added to the conditioned media, this cancer-induced inhibition of differentiation was totally reversed (*A3*). This indicates that cancer cells inhibit adipocyte differentiation, at least in part, by the secretion of IL-11 and TNF-α. *B*, levels of TNF-α (*B1*) and IL-11 (*B2*) in various conditioned media before (*Day 0*) and after (*Day 2*) addition to 3T3-L1 cells in culture. *DMEM*, Control medium; *COC*, differentiating cocktail. *Bars*, SE. *C*, Northern blot analysis demonstrating TNF-α transcripts in T47D breast cancer cells treated with lipopolysaccharide (*LPS*, 1 μg/ml) and TNF-α (10 ng/ml) for 3 h. Under similar treatment conditions, transcripts were not detected in 3T3-L1 murine fibroblasts. The murine RAW264.7 macrophage line was used as a positive control, whereas the U937 human leukemia cell line was used as a negative control. The probe was a full-length murine TNF-α cDNA (the molecular weight of human transcripts was relatively lower, as expected). *D*, immunoreactive TNF-α (*D1*) and IL-11 (*D2*) were readily detectable in malignant epithelial cells but not in fibroblasts. The *Brown* stain indicates immunoreactive cytokines (*arrows*). These representative sections depict similar data observed in breast cancer samples from 15 patients.

medium, because human cells required prolonged exposure to this medium in contrast to 3T3-L1 murine fibroblasts. At the end of coculture experiments, human adipose fibroblasts or 3T3-L1 cells in the bottom plate were evaluated for differentiation to mature adipocytes and for proliferative indices (rate of [^{3}H]thymidine incorporation and flow cytometry) or were harvested for RNA isolation.

Immunohistochemistry. Antihuman IL-II, TNF-α, C/EBPα, C/EBPβ, and C/EBPδ antibodies were purchased from R&D Systems, Inc. The immunohistochemical procedures were performed, as described previously, on 2.5-μm-thick sections mounted on poly-L-lysine-coated slides using the biotin-streptavidin amplified technique with a histone immunostaining kit (Nichirei, Tokyo, Japan). Briefly, this staining procedure was performed as follows: (*a*) routine deparaffinization; (*b*) inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 20 min at 23°C; (*c*) blocking with 1% goat serum for 45 min at 23°C; (*d*) incubation with the primary antibody at 4°C for 18 h; (*e*) incubation with biotinylated goat antirabbit antibody for 30 min at 23°C; (*f*) incubation with peroxidase-conjugated streptavidin for 30 min at 23°C; (*g*) colorimetric reaction with a solution containing 0.05% Tris-HCl (pH 7.6), 0.66 M 3,3′-diaminobenzidine, and 2 M H_2O_2; and (*h*) counterstaining with 1% methyl green.

ELISA Assay. For the determination of IL-11 and TNF-α concentrations in breast cancer cell conditioned media, IL-11 and TNF-α ELISA immunoassay kits (R&D Systems, Inc.) were used according to the instructions supplemented by the vendor. Briefly, samples of media were collected immediately before and 2 days after the addition of these media to 3T3-L1 cells. Samples (100 μl) were assayed in triplicate. After 2–16 h of incubation at room temperature, each well was aspirated. IL-11 or TNF-α conjugates (200 μl) were added to the wells and incubated for 2 h at room temperature. Each well was aspirated. The substrate solution (200 μl) was then added to each well and incubated for 30 min. After adding 50 μl of the stop solution to each well, the absorbance was immediately determined at a wavelength of 450 nm. IL-11 or TNF-α concentrations were calculated by creating standard curves and plotting the mean absorbance for each standard on the Y axis against the concentration on the X axis.

RESULTS

Effects of Tumor Cells on the Differentiation of Fibroblasts to Adipocytes. Confluent 3T3-L1 cells differentiate to mature adipocytes within 4 or 6 days after a 48-h treatment with a mixture including insulin, DEX, and MIX. At this stage, the cells appear rounded and possess numerous large cytosolic lipid spheres, as revealed by Oil Red O staining (Fig. 1*A1*). The effect of the T47D breast cancer cell line on the adipogenic differentiation of the 3T3-L1 cells was evaluated with cocultures or T47D cell conditioned medium. Medium conditioned with T47D breast cancer cells completely inhibited the differentiation of 3T3-L1 cells (Fig. 1*A2*). Upon addition of neutralizing antibodies to human IL-11 and TNF-α, this effect was totally reversed. In other words, neutralizing both of these cytokines reversed the inhibitory effect of breast cancer cells on adipocyte differentiation (Fig. 1*A3*). This reversal was partial when either antibody was used separately and was dose dependent (data not shown). Antihuman IL-2 neutralizing antibodies or the normal goat IgG antibody did not reverse the inhibition, indicating that the effects of anti-IL-11 and TNF-α neutralizing antibodies were specific.

Cocultures of T47D cancer cells also inhibited the differentiation of human adipose fibroblasts completely. Other breast cancer cell lines (MCF-7, SSC202, SSC78, and SSC30) also inhibited the differentiation of both 3T3-L1 murine fibroblasts and human adipose fibroblasts. Moreover, the liver cancer cell line HepG2 and the choricarcinoma cell line JEG3 also inhibited the differentiation of these cells to adipocytes (data not shown). Because all malignant epithelial cells tested inhibited differentiation, normal primary mammary epithelial cell conditioned medium was used to further determine the specificity of inhibition of differentiation. Normal mammary epithelial cells did not inhibit the differentiation of 3T3-L1 cells (data not shown), which demonstrated that the inhibition on differentiation is specific for malignant cells.

Cellular Localization of the Cytokines Inhibitory for Adipocyte Differentiation in the Breast Cancer. Previous experiments have suggested that IL-11and TNF-α mediated the inhibition of adipocyte differentiation by cancer cells. Thus, we determined the cellular origin of these cytokines in breast cancer:

(*a*) Both TNF-α and IL-11 concentrations in TCM were 2.5–3 times those found in media conditioned with control cells. The levels of these cytokines did not increase during the incubation of 3T3-L1 cells, indicating that 60–75% of IL-11 and TNF-α in the medium arises from T47D cells (Fig. 1, *B1* and *B2*).

(*b*) We demonstrated TNF-α transcripts in T47D breast cells but not in 3T3-L1 cells, using Northern blotting (Fig. 1*C*).

(*c*) We determined the *in vivo* cellular distribution of immunoreactive IL-11 and TNF-α in 15 mastectomy specimens. These two cytokines were primarily expressed in all malignant epithelial cells. Less than 25% of fibroblasts contained immunoreactive IL-11 or TNF-α with considerably less staining intensity (Fig. 1*D*).

Expression of PPARγ, C/EBPα, and aP2 in 3T3-L1 Cells Cocultured with Breast Cancer Cells. We determined the levels of transcripts of C/EBPα, C/EBPβ, C/EPBδ, PPARγ, and aP2 by Northern analysis in cocktail-treated 3T3-L1 cells exposed to conditioned media from T47D breast cancer cells (TCM) and HEMC normal breast epithelial cells (NCM). Fig. 2 depicts these results. Treatment with TCM strikingly decreased the transcript levels of C/EBPα and PPARγ in 3T3-L1 cells, whereas it significantly potentiated the effects of the differentiation mixture to induce the expression of C/EBPβ and C/EBPδ during days 4 and 6 after the initiation of treatment. As expected, TCM also inhibited the expression of aP2, which is a marker for adipocyte differentiation (Fig. 2*B*). Upon the addition of a neutralizing antibody against TNF-α, the effects of TCM on C/EBPs and PPARγ expression were reversed completely (Fig. 2*C*), whereas NCM did not change the expression pattern of C/EBPs and PPARγ. This demonstrated that the inhibition caused by TCM on the induction of C/EBPα and PPARγ was specific for malignant epithelial cells. These results were further confirmed by performing human breast tumor immunohistochemistry.

We determined the number and staining intensity of immunoreactive cells for C/EBPα, C/EBPβ, and C/EPBδ in fibroblasts mixed with malignant epithelial cells (intratumoral), within fat 1 cm from the tumor and within fat 2–4 cm from the tumor. An H-scoring system was used to determine the number of immunoreactive fibroblasts, and this is illustrated in Fig. 3. C/EBPα was not detectable in intratumoral fibroblasts located proximal malignant epithelial cells, but it was readily detectable in fibroblasts/adipocytes in fat tissue biopsies distal to the tumor, whereas immunoreactive C/EBPβ and C/EBPδ were present in intratumoral fibroblasts proximal to malignant cells (Fig. 3). These results are in agreement with those of the Northern analysis.

Effects of T47D Breast Cancer Cells on the Proliferation of 3T3-L1 Fibroblasts. We determined whether T47D cancer cells affected the proliferation indices of 3T3-L1 fibroblasts, when these two cell types were cocultured. We did not observe any differences in the DNA histograms of 3T3-L1 cells incubated in the absence or presence of T47D cells using flow cytometry (data not shown). Neither did we see an effect of T47D breast cancer cells on the [^{3}H]thymidine incorporation into 3T3-L1 cells (data not shown). Thus, we conclude that breast cancer cells induce accumulation of fibroblasts in the tumor tissue by the inhibition of differentiation of these cells to mature adipocytes but not by promotion of their proliferation.

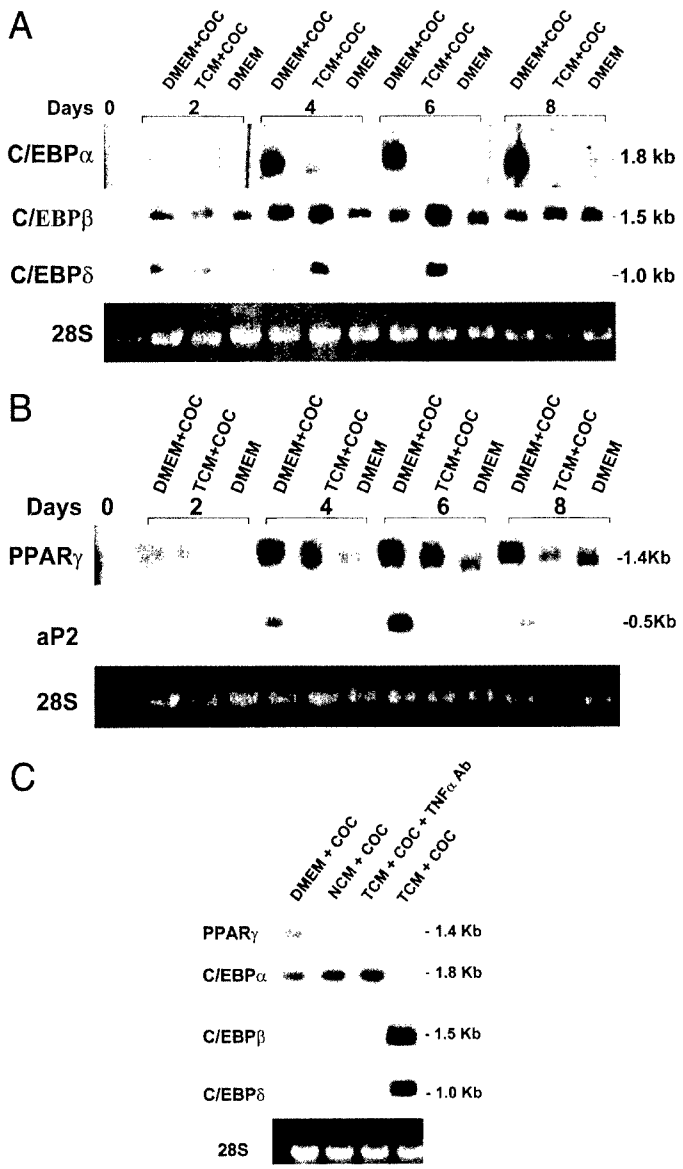


Fig. 2. Northern blot showing the levels of transcripts of C/EBPα, C/EBPβ, C/EBPδ (*A*), PPARγ, and aP2 (*B*) in 10 μg of total RNA from 3T3-L1 cells incubated under conditions explained in the legend of Fig. 1*B*. The 28S RNA fraction was included to demonstrate the presence of comparable amounts of RNA in each lane. *DMEM*, control medium; *COC*, differentiating cocktail. T47D cells (TCM) inhibited expression of C/EBPα and PPARγ and enhanced expression C/EBPβ or C/EBPδ in 3T3-L1 cells after exposure to the differentiating cocktail (*COC*). T47D cells (TCM) also suppressed the expression of aP2, a marker of differentiated adipocytes. *C*, on day 6. NCM did not change the expression patterns of C/EBPs and PPARγ. Furthermore, the addition of an anti-TNFα neutralizing antibody completely abolished the influence of TCM on C/EBPs and PPARγ, indicating the essential role of TNF-α in mediating the effects of TCM. Please note that Northern blots in *A–C* represent separate experiments, and exposure times of autoradiographs were different.

DISCUSSION

Peri- and intratumoral fibroblasts provide structural support to tumor tissue, and secretory products of fibroblasts may promote tumor growth. We herein demonstrated that malignant breast epithelial cells actively participate in the process of accumulation of stromal fibroblasts in and around the tumor tissue (*i.e.*, desmoplastic reaction). Secretory products of cancer cells prevent the differentiation of fibroblasts to adipocytes and, in fact, may even reverse adipocyte differentiation. We also demonstrated that tumor-derived cytokines act on adjacent adipose stroma by down-regulating the expression of adipogenic factors such as the C/EBPα and PPARγ. This study also provides evidence that breast cancer cells (or their secretory products) do not induce proliferation of fibroblasts. Thus, inhibition of differentiation seems to be the major mechanism responsible for desmoplastic reaction.

We chose to study IL-11 and TNF-α as the malignant epithelial cell-derived factors, because the potent antiadipogenic effects of these two cytokines have been well established (27–29). In our hands, the combined effects of IL-11 and TNF-α were sufficient to inhibit adipocyte differentiation completely. Most importantly, both cytokines are expressed in abundant levels in malignant epithelial cells. The effects of these cytokines are specific for malignant cells, because other cytokines or benign breast epithelial cells did not inhibit adipocyte differentiation.

Differentiation of fibroblastic cells to adipocytes appears to be primarily controlled by a cascade of adipogenic factors. C/EBPβ and C/EPBδ appear to mediate the earlier phase of the differentiation program. PPARγ, a nuclear hormone receptor, is expressed next in the differentiation process and becomes adipogenic after binding to its synthetic (BRL49653; Ref. 26) and natural (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) ligands (23). PPARγ binds to its response elements in the promoters of its target genes. To recognize a PPARγ response element, PPARγ must form a heterodimer with retinoid X receptor α, a basic-leucine zipper transcription factor. On the other hand, C/EBPα is not expressed until relatively late in the differentiation process. It binds to and transactivates the promoters of a number of adipocyte-specific genes. Thus, breast cancer cells seem to exert their inhibitory effect on relatively later stages of the differentiating process, *i.e.*, inhibition of the expression of PPARγ and C/EBPα. This is consistent with the isolated effects of TNF-α, which has been shown to inhibit the expression of PPARγ and C/EBPα in 3T3-L1 cells and fetal brown adipocytes (27, 28).

This report lays the groundwork for the mechanism of desmoplastic reaction as an epithelial-stromal interaction in the breast cancer. Further studies are required to identify other key molecules in the cancer-mediated inhibition of adipocyte differentiation. Two candidate substances are CHOP, a transcription factor that acts as a negative dominant regulator of adipocyte differentiation, and Pref-1, which is a transmembrane protein with epidermal growth factor-like motifs and another negative regulator of adipocyte differentiation (30). The determination of the roles of these candidate substances will further increase our understanding of the epithelial-stromal interactions in the

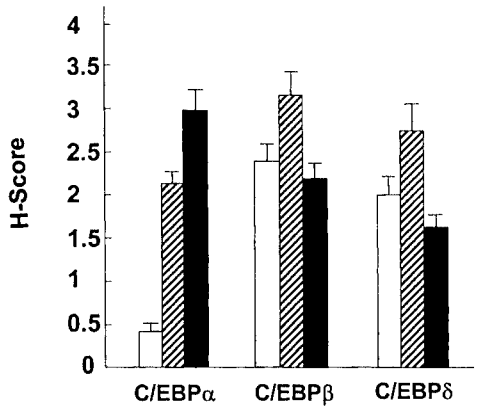


Fig. 3. Expression patterns of C/EBPs in mastectomy specimens from 10 patients with breast cancer. Immunoreactive proteins were scored in the three biopsies (intratumoral; fat, 1 cm to tumor; and fat, 2–4 cm to tumor; from each of the 10 mastectomy specimens). H-scoring was used in the following manner: 0, 0–5% fibroblasts are positive; 1, 6–25% fibroblasts are positive; 2, 26–50% fibroblasts are positive; 3, 51–75% fibroblasts are positive; and 4, 76–100% fibroblasts are positive. Immunoreactive C/EBPα is not detected in the fibroblasts within the breast tumor but is readily detectable in the fibroblasts/adipocytes distant to the tumor. Immunoreactive C/EBPβ and C/EBPδ are readily detectable in all of the three biopsy sites. □, intratumoral; ▨, adipose tissue (1 cm); ■, adipose tissue (2–4 cm). *Bars*, SE.

breast cancer. Finally, we recently showed that cancer cell-induced up-regulation of C/EBPβ mediates aromatase overexpression in adipose fibroblasts (31).

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REFERENCES

1. Haagensen, C. D. Diseases of the Breast, Ed. 3, pp. 635–641. Philadelphia: W. B. Saunders Co., 1986.
2. Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. Endocr. Rev., *16:* 559–589, 1995.
3. Haslam, S. Z. Stromal-epithelial interactions in normal and neoplastic mammary gland. *In:* M. E. Lippman and R. B. Dickson (eds.), Regulatory Mechanisms in Breast Cancer, pp. 401–420. Boston: Kluwer Academic Publishers, 1991.
4. Bulun, S. E., and Simpson, E. R. Breast cancer and expression of aromatase in breast adipose tissue. Trends Endocrinol. Metab., *5:* 113–120, 1994.
5. Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E. F. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J. Clin. Invest., *84:* 1663–1670, 1989.
6. Adams, M., Prins, J., Holder, J., Sanders, L., Smith, S., O'Rahilly, S., and Chatterjee, V. K. K. Thiazolidinediones promote human pre-adipocyte differentiation and activate hPPARγ. Endocr. Soc., *78:* 137, 1996.
7. Bulun, S. E., Prince, T. M., Mahendroo, M. S., Aitken, J., and Simpson, E. R. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. J. Clin. Endocrinol. Metab., *77:* 1622–1628, 1993.
8. Ackerman, G. E., Smith, M. E., Mendelson, C. R., MacDonald, P. C., and Simpson, E. R. Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. J. Clin. Endocrinol. Metab., *53:* 412–417, 1981.
9. Price, T., Aitken, J., Head, J., Mahendroo, M. S., Means, G. D., and Simpson, E. R. Determination of aromatase cytochrome P450 messenger RNA in human breast tissues by competitive polymerase chain reaction (PCR) amplification. J. Clin. Endocrinol. Metab., *74:* 1247–1252, 1992.
10. Bulun, S. E., Sharda, G., Rink, J., Sharma, S., and Simpson, E. R. Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. J. Clin. Endocrinol. Metab., *81:* 1273–1277, 1996.
11. Nichols, J. E., Bulun, S. E., and Simpson, E. R. Effects of conditioned medium from different cultured cell types on aromatase expression in adipose stromal cells. J. Soc. Gynecol. Investig., *2:* 45–50, 1995.
12. Crichton, M. B., Nichols, J. E., Zhao, Y., Bulun, S. E., and Simpson, E. R. Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. Mol. Cell. Endocrinol., *118:* 215–220, 1996.
13. Zhao, Y., Nichols, J. E., Bulun, S. E., Mendelson, C. R., and Simpson, E. R. Aromatase *P450* gene expression in human adipose tissue: role of a Jak/STAT pathway in regulation of the adipose-specific promoter. J. Biol. Chem., *270:* 16449–16457, 1995.
14. Reed, M. J., Topping, L., Coldham, N. G., Purohit, A., Ghilchik, M. W., and James, V. H. T. Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors. J. Steroid Biochem. Mol. Biol., *44:* 589–596, 1993.
15. Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C., and Rubin, C. S. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3–L1 adipocytes. J. Biol. Chem., *263:* 9402–9408, 1988.
16. Boney, C. M., Smith, R. M., and Gruppuso, P. A. Modulation of insulin-like growth factor I mitogenic signaling in 3T3–L1 preadipocyte differentiation. Endocrinology, *139:* 1638–1644, 1998.
17. Adachi, H., Kurachi, H., Homma, H., Adachi, K., Imai, T., Morishige, K., Matsuzawa, Y., and Miyake, A. Epidermal growth factor promotes adipogenesis of 3T3–L1 cell *in vitro*. Endocrinology, *135:* 1824–1830, 1994.
18. Schmidt, W., Poll-Jordan, G., and Loffler, G. Adipose conversion of 3T3–L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP. J. Biol. Chem., *265:* 15489–15495, 1990.
19. Wu, Z., Xie, Y., Bucher, N. L. R., and Farmer, S. R. Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. Genes Dev., *9:* 2350–2363, 1995.
20. Cao, Z., Umek, R. M., and McKnight, S. L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3–L1 cells. Genes Dev., *5:* 1538–1552, 1991.
21. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. PPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev., *8:* 1224–1234, 1994.
22. Hu, E., Tontonoz, P., and Spiegelman, B. M. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc. Natl. Acad. Sci. USA, *92:* 9856–9860, 1995.
23. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. 15-Deoxy-$\delta^{12,\,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell, *83:* 803–812, 1995.
24. Tontonoz, P., Hu, E., and Spiegelman, B. M. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell, *79:* 1147–1156, 1994.
25. Zhang, B., Berger, J., Szalkowski, D., White-Carrington, S., and Moller, D. E. Negative regulation of *PPAR*γ gene expression underlies the anti-adipogenic effects of TNFα. Proc. 10th Int. Congress Endocrinol., *10:* 141, 1996.
26. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. Z., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem., *270:* 12953–12956, 1995.
27. Zhang, B., Berger, J., Hu, E., Szalkowski, D., White-Carrington, S., Spiegelman, B. M., and Moller, D. E. Negative regulation of peroxisome proliferator-activated receptor-γ gene expression contributes to the antiadipogenic effects of tumor necrosis factor-α. Mol. Endocrinol., *10:* 1457–1466, 1996.
28. Valverde, A. M., Teruel, T., Navarro, P., Benito, M., and Lorenzo, M. Tumor necrosis factor-α causes insulin receptor substrate-2-mediated insulin resistance and inhibits insulin-induced adipogenesis in fetal brown adipocytes. Endocrinology, *139:* 1229–1238, 1998.
29. Kawashima, I., Ohsumi, J., Mita-Honjo, K., Shimoda-Takano, K., Ishikawa H, Sakakibara, S., Miyadai, K., and Takiguchi, Y. Molecular cloning of cDNA encoding adipogenesis inhibitory factor and identity with interleukin-11. FEBS Lett., *283:* 199–202, 1991.
30. Smas, C. M., and Sul, H. S. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell, *73:* 725–734, 1993.
31. Zhou, J., Gurates, B., Yang, S., Sebastian, S., and Bulun, S. E. Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein β. Cancer Res., *61:* 2328–2334, 2001.

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Malignant Breast Epithelial Cells Stimulate Aromatase Expression via Promoter II in Human Adipose Fibroblasts: An Epithelial-Stromal Interaction in Breast Tumors Mediated by CCAAT/Enhancer Binding Protein β[1]

Jianfeng Zhou, Bilgin Gurates, Sijun Yang, Siby Sebastian, and Serdar E. Bulun[2]

Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois 60612

ABSTRACT

Expression of aromatase P450 (P450arom), which catalyzes the formation of estrogens, is aberrantly increased in adipose fibroblasts surrounding breast carcinomas, giving rise to proliferation of malignant cells. Aromatase in human adipose tissue is primarily expressed in undifferentiated fibroblasts under the control of several distinct and alternatively used P450arom promoters. In tumor-free breast adipose tissue, P450arom is usually expressed at low levels via a distal promoter (I.4), whereas in the breast adipose tissue bearing a tumor, P450arom is increased through the activation of two proximal promoters, II and I.3. Because the *in vivo* activation of P450arom promoter II is a key event responsible for aberrantly high P450arom expression in breast tumors, we studied the molecular basis for the enhancement of P450arom promoter II using human adipose fibroblasts (HAFs) in primary culture treated with T47D breast cancer cell-conditioned medium (TCM) as a model system. Upon treatment with TCM, HAFs displayed a striking induction of P450arom mRNA levels via promoter II usage. This effect appeared to be specific for malignant breast epithelial cells, because conditioned media from breast cancer cell lines T47D and MCF-7 induced promoter II activity, whereas normal breast epithelial cells or liver or prostate cancer cell lines did not produce such an effect. Although treatment with a cyclic AMP analogue also caused a switch in the promoter use from I.4 to II in cultured HAFs, TCM-induced promoter II use was found to be mediated via a cyclic AMP-independent pathway. Use of serial deletion mutants of the promoter II 5′-flanking sequence revealed the presence of critical *cis*-acting elements in the −517/−278 bp region, which regulate the baseline activity. TCM caused a 5.7-fold induction of the −517-bp promoter II construct, whereas site-directed mutagenesis of a CCAAT/enhancer binding protein (C/EBP) binding site (−317/−304 bp) abolished both baseline and TCM-induced activities. Ectopic expressions of C/EBPα and C/EBPβ, but not C/EBPδ, significantly induced promoter II activity. Moreover, we demonstrated the presence of both C/EBPβ and C/EBPδ but not C/EBPα in a DNA-protein complex formed by the nuclear extract from TCM-treated HAFs and a probe containing this critical C/EBP binding element (−317/−304 bp). Finally, treatment of HAFs with TCM strikingly induced C/EBPβ expression, whereas this did not affect the levels of C/EBPα or C/EBPδ transcripts. In conclusion, malignant breast epithelial cells secrete factors, which induce aromatase expression in adipose fibroblasts via promoter II. This is, at least in part, mediated by a TCM-induced up-regulation and enhanced binding of C/EBPβ to a promoter II regulatory element.

INTRODUCTION

The conversion of C_{19} steroids to estrogens by P450arom[3] takes place in a number of human cells, *e.g.*, the ovarian granulosa cell (1), skin, and adipose fibroblasts (2, 3). Aromatase expression in the adipose tissue is limited to fibroblasts and is not detected in significant quantities in the fully differentiated and lipid-filled adipocytes (2, 3). Aromatase activity in adipose fibroblasts has long been implicated in the pathophysiology of breast cancer growth (4–7). Estrogen produced in breast adipose tissue acts locally to promote the growth of tumor (8). Thus, the relationship between adipose stroma and breast cancer is unique in that the adipose fibroblast provides structural and functional support for cancer growth. O'Neill *et al.* (6) demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor. Subsequently, we found the highest levels of P450arom transcripts in adipose tissue from the quadrant bearing a tumor (7). In the same study, tumor-bearing quadrants contained the highest fibroblast:adipocyte ratios. It follows then that the breast quadrant with the highest fibroblast content contains the highest levels of P450arom transcripts. The clinical relevance of these observations has been exemplified by the successful treatment of breast carcinomas with potent aromatase inhibitors (9–11).

Expression of the human P450arom (*CYP19*) gene is under the control of several distinct and partly tissue-specific promoters (12, 13). Three of these promoters (I.4, I.3, and II) are used in adipose tissue. Interestingly, in disease-free breast adipose tissue, P450arom is usually expressed at low levels via a distal promoter (I.4), whereas in the adipose tissue of the breast bearing a tumor, P450arom expression is increased through the activation of two proximal promoters, II and I.3 (14–16). In addition to these *in vivo* observations, treatments of HAFs in culture with various hormones switch promoter use. For example, glucocorticoids plus cytokines induce P450arom expression via promoter I.4 in cultured primary HAFs, whereas treatment with a cAMP analogue switches the promoter use to II and I.3 (12, 13). We hypothesized that malignant breast epithelial cells interact with the surrounding adipose tissue fibroblasts to activate promoters II and I.3. The data presented in this report will serve to reconcile the *in vivo* and *in vitro* observations summarized above (12–14). We report a novel epithelial-stromal interaction, which favors the induction of P450arom expression in HAFs by malignant epithelial cells via promoter II.

We and others have shown previously that breast cancer cells could stimulate aromatase expression in HAFs, which was suggestive of cross-talk between malignant epithelial cells and surrounding HAFs to favor estrogen production in breast tumors (17–19). We demonstrated recently that medium conditioned with malignant epithelial cells inhibited the differentiation of HAFs to mature adipocytes via the suppression of the essential adipogenic transcription factors C/EBPα and PPARγ. C/EBPβ and C/EBP/δ, on the other hand, were up-regulated in these undifferentiated murine fibroblasts treated with TCM (20). TCM-induced decreases in C/EBPα or PPARγ were

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[2] To whom requests for reprints should be addressed, at Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, 820 South Wood Street, M/C 808, Chicago, IL 60612. Phone: (312) 996-8197; Fax: (312) 996-4238; E-mail: sbulun@uic.edu.

[3] The abbreviations used are: P450arom, aromatase P450; HAF, human breast adipose fibroblast; cAMP, cyclic AMP; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; TCM, T47D cell conditioned medium; Bt_2cAMP, dibutyryl cAMP; PDA, phorbol diacetate; DEX, dexamethasone; FBS, fetal bovine serum; NCM, normal mammary epithelial cell conditioned medium; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SF, steroidogenic factor; CREB, cAMP response element binding protein; EMSA, electrophoresis mobility shift analysis; TNF, tumor necrosis factor; IL, interleukin; CMV, cytomegalovirus.

sufficient to completely inhibit adipogenic differentiation of 3T3-L1 cells in our hands (20). This was in agreement with reports published previously (21, 22). On the other hand, we were intrigued by the TCM-induced increases in C/EBPβ and C/EBP/δ mRNA levels in 3T3-L1 murine cells (20). In contrast to C/EBPα or PPARγ, ectopic expressions of C/EBPβ or C/EBPδ were not sufficient to induce adipocyte differentiation in the absence of C/EBPα or PPARγ (23). Thus, we hypothesize that breast cancer-induced increases in C/EBPβ or C/EBP/δ levels do not affect adipocyte differentiation but may serve to increase aromatase expression in adipose fibroblasts surrounding the cancer. We used herein a model whereby human TCM is added to primary HAFs to understand the roles of C/EBP isoforms in the up-regulation of aromatase expression in undifferentiated fibroblasts. We chose to study the activation of promoter II, because work from three different laboratories demonstrated that the activity of this promoter was up-regulated *in vivo* in breast stroma bearing a carcinoma (14–16).

MATERIALS AND METHODS

Cell Cultures. Human adipose tissues were obtained at the time of surgery from women undergoing reduction mammoplasty following a protocol approved by the Institutional Review Board for Human Research of the University of Illinois at Chicago. For primary HAF cultures, adipose tissues were minced and digested with collagenase B (1 mg/ml) at 37°C for 2 h. Single-cell suspensions were prepared by filtration through a 75-μm sieve. Fresh cells were suspended in DMEM/F12 containing 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. Twelve to 24 h after the attachment of fibroblasts, culture medium was removed, and cell medium was changed at 48-h intervals until the cells became confluent. Before total RNA or nuclear proteins were extracted from HAFs, these cells were cultured in either serum-free DMEM/F12, DMEM/F12 containing 10% FBS, serum-free DMEM/F12 containing Bt_2cAMP (0.5 mM) together with PDA (100 nM), DMEM/F12 containing 10% FBS plus DEX (250 nM), or DMEM/F12 conditioned with malignant or benign cells. All treatments were continued for 48 h.

T47D cells purchased from American Type Culture Collection (Rockville, MD) were initially grown in RPMI 1640 with 10% FBS containing 0.02 mM HEPES, whereas MCF-7 cells, prostate cancer cell line PC-3, and hepatocellular carcinoma cell line HepG2 (American Type Culture Collection) were grown in MEM with 10% FBS. Human normal mammary epithelial cells purchased from Clonetics, Inc. (Walkersville, MD) were grown in fully supplemented MEGM medium (Clonetics). Before shipment, these cells were passed twice and demonstrated to contain immunoreactive cytokeratins 14 and 18. In our hands, these cells were alive and dividing every 48–72 h. Cell-conditioned media from T47D, MCF-7, PC-3, HepG2, or normal mammary epithelial cells were collected to be used subsequently as treatments on HAFs. To collect conditioned media, cells were initially grown to confluence and switched to DMEM/F12 for a 12-h washout period; then, cells were incubated in DMEM/F12 for 24 h to allow accumulation of secreted factors in the medium.

RT-PCR Amplification. Amplification of the untranslated 5′ ends of P450arom transcripts from HAFs under various treatments was accomplished with exon-specific oligonucleotide pairs as described below. Five μg of DNase I-treated total RNA were used for reverse transcriptase reaction. Five μl of reverse transcriptase mixture were amplified using PCR. For the amplification of total P450arom transcripts, 5′-end sense primer from coding exon II (5′-TTG GAA ATG CTG AAC CCG AT-3′) and 3′-end antisense primer complimentary to coding exon III (5′-CAG GAA TCT GCC CTG GGG AT-3′) were used. To amplify promoter-specific 5′-untranslated sequences, primers for promoter II-specific sequence (5′-GCA ACA GGA GCT ATA GAT-3′) and exon I.4 (5′- GTA GAA CGT GAC CAA CTG G-3′) were used as 5′-end sense primers, together with an antisense primer complimentary to the coding exon III (5′-ATT CCC ATG CAG TAG CCA GG-3′). PCR conditions were as follows: denaturing at 95°C for 30 s, annealing at 55°C for amplification of promoter II-specific sequence or 58°C for amplification of exon I.4 and the coding region for 40 s, and extension at 72°C for 40 s for 30 cycles. GAPDH was chosen as an endogenous marker to check the integrity of cDNA. A 5′-end sense primer (5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′) and a 3′-end antisense primer (5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′) were used for amplifying a 306-bp-long sequence in GAPDH mRNA. PCR conditions were the same as those used for amplification of promoter II-specific fragments, except for the number of cycles (21) and the quantity of reverse transcriptase mixture (0.5 μl). This RT-PCR method was described previously in greater detail (14).

Determination of Intracellular cAMP. HAFs were plated in six-well, 35-mm culture dishes. After reaching confluence, HAFs were cultured either in serum-free DMEM/F12, DMEM/F12 containing 10% FBS, DMEM/F12 containing 10% FBS and forskolin (10 μM), DMEM/F12 containing 10% FBS and DEX (250 nM), or T47D cell conditioned DMEM/F12. Measurements were performed in triplicate replicates, and treatments were carried for 0, 12, 24, and 48 h. HAFs were lysed in a 0.1 M HCl solution after the removal of medium. Cell lysis mixture was centrifuged, and the supernatant was then used directly in the cAMP assay using Direct Cyclic AMP Enzyme Immunoassay kit (Assay Design, Inc., Ann Arbor, MI), following the protocol supplied by the vendor. Briefly, 50 μl of the pink-neutralizing reagent were added into each well, except for the total activity and blank wells. Samples (100 μl) were then added to appropriate wells. Fifty μl of the conjugate were added into each well, followed by the addition of 50 μl of the yellow antibody. After incubating at room temperature for 2 h on a shaker at 500 rpm, the plate was washed three times with 200 μl of washing buffer, followed by the addition of substrate solution 200 μl to each well. The stop solution (50 μl) was then added to each well, and the absorbance was read at 405 nm with correction to 570 nm. Results were obtained by plotting on the standard curve.

Transient Transfections and Luciferase Assays. HAFs in primary culture were transfected using Lipofectamine Plus (Life Technologies, Inc., Grand Island, NY) with the following plasmids: (*a*) 1 μg of modified PGL_3-Basic Luciferase reporter plasmid that contains serial deletion mutants of P450arom promoter II; (*b*) 0.2 μg of pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA), which contains the cDNA of either C/EBPα (human), C/EBPβ (rat) or C/EBPδ (rat); and (*c*) 5 ng of pRL-CMV *Renilla* luciferase control reporter vectors that contain the cDNA encoding *Renilla* luciferase (Promega Corp., Madison, WI) as an internal control for transfection efficiency. The day before transfection, HAFs in primary culture were seeded into 35-mm dishes at 2×10^5 cell/dish. The transfection solution was made of 200 μl of OPTI-MEM I reduced-serum medium containing PLUS reagent (8 μl), precomplexed DNA (1.2 μg), and 5 μl of Lipofectamine reagent. After transfection for 6 h in transfection solution at 37°C in 5% CO_2, medium was changed to antibiotic-free DMEM/F12 containing 10% FBS for overnight recovery. Cells were then switched to medium conditioned by normal breast epithelial cells or T47D cells for another 48 h. Luciferase and *Renilla* luciferase assays were performed using a dual-luciferase reporter assay system kit (Promega). Results are presented as the average of data from triplicate replicates and expressed as the ratio to the internal standard *Renilla* luciferase. The empty luciferase vector PGL_3-Basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed as multiples of the PGL_3-Basic vector.

Northern Blotting. Total RNA was isolated from HAFs in primary culture growing in: (*a*) DMEM/F12; (*b*) normal breast epithelial cell conditioned medium; or (*c*) TCM. Twenty μg of total RNA were used. cDNA probes for C/EBPδ, C/EBPβ, and C/EBPα were prepared from plasmids kindly provided by Drs. Steve McKnight (University of Texas Southwestern Medical Center, Dallas, TX), Gokhan Hotamisligil (Harvard Medical School, Boston, MA), and Gretchen Darlington (Baylor College of Medicine, Houston, TX).

Site-directed Mutagenesis. To generate serial plasmids bearing mutated consensus-binding sequences for transcription factors of C/EBPs, SF-1 and CREB, site-directed mutagenesis was performed using the GeneEditor *in vitro* site-directed mutagenesis system (Promega), per the manufacturer's instructions. A −517-bp promoter II/PGL_3-Basic construct containing wild-type −517/−16 bp of P450arom promoter II 5′-flanking DNA was used as a template for site-directed mutagenesis. Briefly, DNA template (0.5 pmol) was denatured and annealed with mutagenic and selection oligonucleotides. Mutant strand was synthesized in the reaction mixture containing 1× synthesis buffer, 5 units of T4 DNA polymerase, and 2 units of T4 DNA ligase at 37°C for 90 min. The mutagenesis reaction mixture was then used to transform BMH 71-18 *mut*S competent cells. These transformed competent cells were incubated in a medium containing GeneEditor antibiotic selection mix overnight to select the desired mutant plasmids. The plasmids isolated from the BMH 71-18 *mut*S

were transformed into JM109 competent cells. The transformed JM109 competent cells were grown overnight on the LB plates containing ampicillin and GeneEditor antibiotic selection mix to further select the mutated plasmids. The mutation of binding consensus was confirmed by DNA sequencing. Consensus binding sequences for mutation and primers used were depicted in Table 1.

EMSA. The nuclear extracts used for EMSA were prepared as described previously (24). Briefly, cells were grown to confluence and cultured in either DMEM/F12 only or T47D cell conditioned DMEM/F12 for 48 h. Cells were then scraped from the dishes. The cell pellet was resuspended in cold buffer A [10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 9.5 mM DTT, 10 μg/ml leupeptin, 100 μg/ml pepstatin, 2 μg aprotinin, 0.5 mM and phenylmethylsulfonyl fluoride]. The cell pellets were homogenized on ice. Once >90% of the cell membranes were broken, the lysate was centrifuged for 2 min at 700 × *g*. After the supernatant was removed, the nuclear pellet was resuspended in buffer C [20 mM HEPES (pH 7.4), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 20% glycerol] and incubated on ice for 30 min with intermittent mixing. After centrifugation at 60,000 rpm for 5 min at 4°C, the supernatant was snap-frozen in liquid nitrogen. Protein concentrations were determined by a modified Bradford assay (Bio-Rad, Hercules, CA), and nuclear extracts were stored at −80°C.

Double-stranded oligonucleotides were obtained through annealing sense and antisense sequences. The double-stranded oligonucleotide probes were end-labeled with [γ-^{32}P]ATP using T4 kinase. EMSAs were performed as described previously (24). Briefly, 5 μg of nuclear extract were incubated with the radiolabeled double-stranded oligonucleotide probe for 15 min at room temperature in a reaction buffer containing 20 mM HEPES (pH 7.6), 75 mM KCl, 0.2 mM EDTA, 20% glycerol, and 2 μg of poly(deoxyinosinic-deoxycytidylic acid) as a nonspecific competitor. Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels. EMSAs were performed after the addition of 0.5 μl of an antibody against C/EBPα, C/EBPβ, C/EBPδ, or CREB to the binding reaction, followed by a 30-min incubation on ice before electrophoresis. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We used the following double-stranded probes. C/EBP binding site probe (5′-GAA GAA GAT TGC CTA AAC AA-3′) represents an identical 20-bp-long sequence (−303/−322) in the promoter II regulatory region of the P450arom gene. Mutated C/EBP binding site probe (5′-GAA GAA Gcc cGC CTg gtC AA-3′) contains a mutated version of C/EBP binding motif that does not interact with any of the C/EBP isoforms.

RESULTS

Aromatase Expression in HAFs Is Stimulated by Breast Cancer Cell Conditioned Medium via the P450arom Promoter II. Aromatase expression in human tissues is under the control of alternatively used and partially tissue-specific promoters. The coding region of P450arom transcripts and, thus, the translated protein, however, are identical in each tissue site of expression. In the breast adipose tissue of disease-free women, P450arom expression is expressed at low levels via a distal promoter I.4, whereas in the adipose tissue of breast bearing a tumor, P450arom expression is increased through activation of an ovarian-type proximal promoter II (14–16). We attempted to verify these *in vivo* data by the following *in vitro* experiments. Because the use of each alternative promoter gives rise to a P450arom transcript with an untranslated 5′-end unique for that particular promoter, we used exon-specific RT-PCR to determine total and promoter-specific P450arom transcript levels in HAFs in primary culture treated with TCM. As expected, Bt_2cAMP plus PDA stimulated P450arom transcript levels primarily via activation of promoter II, whereas DEX plus serum activated promoter I.4. Most importantly, we found that TCM stimulated P450arom transcript levels via P450arom promoter II activation, which was demonstrated previously *in vivo* in adipose tissue of the breast bearing a tumor (Fig. 1*A*). To address the specificity of effects of T47D cells on HAFs, we used media conditioned with either the MCF-7 breast cancer cell line or normal breast epithelial cells (NCM) to treat HAFs. Medium conditioned with MCF-7 cells but not with normal epithelial cells induced P450arom transcript levels via promoter II (Fig. 1*B*). Furthermore, other malignant cell lines HepG2 and PC-3 failed to activate P450arom gene transcription via promoter II, which demonstrated that the stimulatory effect produced by T47D and MCF7 cells was specific for breast cancer (Fig. 1*C*). These results demonstrate that malignant breast epithelial cells in culture produce specific factors, which stimulate aromatase expression via promoter II.

Activation of P450arom Promoter II by Breast Cancer Cell Conditioned Medium Is Not cAMP Dependent. Because both TCM and cAMP analogues induce aromatase expression in HAF via promoter II, we sought to determine whether this effect of TCM is mediated via increased formation of cAMP in HAFs. Therefore, we first measured the intracellular levels of cAMP in HAFs treated with forskolin, FBS, FBS plus DEX, or TCM. Contrary to our expectations, treatment with TCM decreased intracellular levels of cAMP at 12-, 24-, and 48-h time points (Fig. 2*A*). FBS or FBS plus DEX also decreased cAMP levels, whereas treatment with the adenylate cyclase inducer forskolin (10 μm) gave rise to a striking increase in cAMP levels in HAFs (positive control; Fig. 2*A*). On the other hand, addition of the adenylate cyclase inhibitor SQ 22,536 to the culture medium 0.5 h before the treatment with TCM for 48 h did not inhibit promoter II activation (negative control; Fig. 2*B*). These results indicate that the activation of P450arom promoter II by TCM is mediated via a cAMP-independent pathway.

Regulation of P450arom Promoter II Activity in Primary HAFs. We determined the genomic regions critical for the regulation of baseline levels of promoter II activity in HAFs (Fig. 3). Use of serial deletion mutants of promoter II fused to luciferase reporter gene demonstrated that the −517/−278-bp region contained critical stimulatory elements (Fig. 3).

The C/EBP Binding Sequence (−317/−304 bp) Is Essential for the Breast Cancer Cell-induced Activation of Promoter II. We identified two C/EBP binding sites in the −517/−278 bp region using

Table 1 *Primers used for site-directed mutagenesis*

Mutated consensus sequence[a]	5′-Phosphorylated and mutagenic primers[a]
C/EBP binding site (−350/−337 bp) TTGTTTTGAAATT→TTGTcccGAgggT	5′-GGG AGA TTG CCT TTT TGT ccc GAA ATT GAT TTG GCT TC-3′ 5′-ATT GCC TTT TTG Tcc cGA ggg TGA TTT GGC TTC AAG GG-3′
C/EBP binding site (−317/−304 bp) AGATTGCCTAAACA→AgcccGCCTggtCA	5′-TGG CTT CAA GGG AAG AAG ccc GCC TAA ACA AAA CCT GCT G-3′ 5′-CAA GGG AAG AAG ccc GCC Tgg tCA AAA CCT GCT GAT GAA G-3′
SF-1 binding site (−263/−251 bp) ATGAGCTTTATTT→ATGgGaaTTATTT	5′-GAC TCC ACC TCT GGA ATG gGa aTT ATT TTC TTA TAA TTT GGC-3′
SF-1 binding site (−136/−124 bp) AGGTCAGAAA→cccTCAGAAA	5′-GGA ACC TGA GAC TCT ACC Acc cTC AGA AAT GCT GCA ATT CAA GC-3′
CRE (−211/−197 bp) TGCACGTCACTCT→TGgAatTCACTCT	5′-GGC TTT CAA TTG GGA ATG gAa tTC ACT CTA CCC ACT CAA GGG CA-3′

[a] Lowercase represents the mutated base pairs.

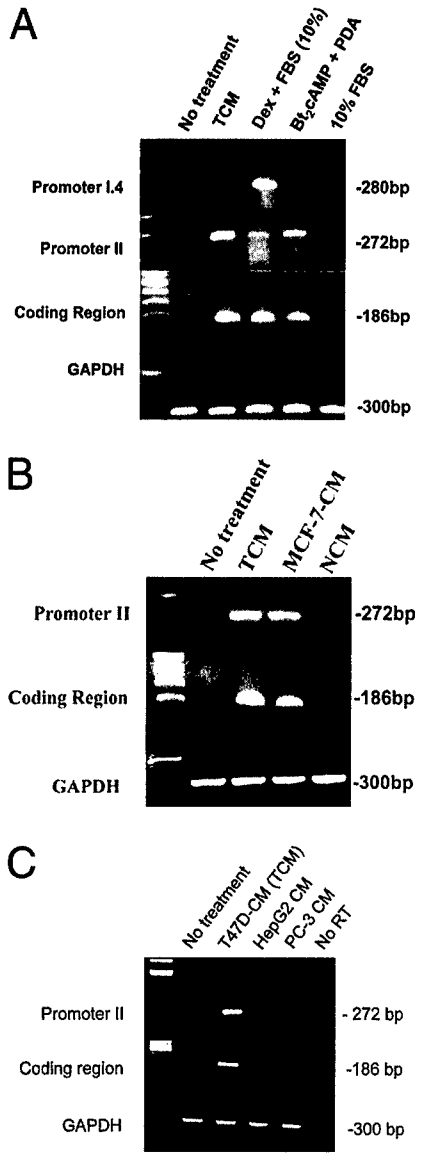

Fig. 1, *A* and *B*, breast cancer cells stimulate P450arom expression via promoter II in primary HAFs. HAFs in primary culture were incubated under various conditions for 48 h [*No treatment*, serum-free DMEM/F12; *TCM; DEX + FBS (10%)*, DEX (250 nM) and FBS; $Bt_2cAMP + PDA$, 0.5 mM Bt_2cAMP + 100 nM PDA; *10% FBS*, DMEM/F12 + 10% FBS; *MCF-7-CM*, MCF-7 cell conditioned medium; *NCM*]. Total RNA was subjected to exon-specific RT-PCR to amplify promoter-specific untranslated first exons. GAPDH was amplified to control the integrity of RNA. *A*, total P450arom transcript levels were up-regulated by treatments with T47D-CM and Bt_2cAMP + PDA via the use of promoter II. DEX + FBS treatment, on the other hand, robustly induced P450arom transcripts via another promoter, promoter I.4. *B*, medium conditioned by another breast cancer cell line, MCF-7, also induced P450arom transcripts via promoter II usage, whereas NCM did not induce P450arom transcript levels at all. *C*, media conditioned by prostate cancer cell line PC-3 and hepatocellular carcinoma cell line HepG2 failed to induce promoter II or the levels of total P450arom transcripts (*Coding region*).

the TFSEARCH database (Fig. 4).[4] The −278/−100-bp region contains two SF-1 sites and a CRE. One of these SF-1 binding sites (−136/−124 bp) and CRE (−211/−197 bp) were shown previously to be critical for cAMP-induced promoter II activity in ovarian granulosa cells and endometriosis-derived stromal cells (24, 25). We determined the effect of TCM on the activity of the −517-bp promoter II/luciferase construct, because this construct showed the highest baseline activity (Fig. 3). Treatment with TCM for 48 h induced the activity of the −517-bp construct by 5.7-fold (Fig. 5). Site-directed mutagenesis of five potentially important *cis*-acting elements demonstrated that CRE (−211/−197 bp) and a C/EBP binding site (−317/−304 bp) were essential for TCM induction of promoter II activity. In particular, mutation of the −317/−304-bp C/EBP binding

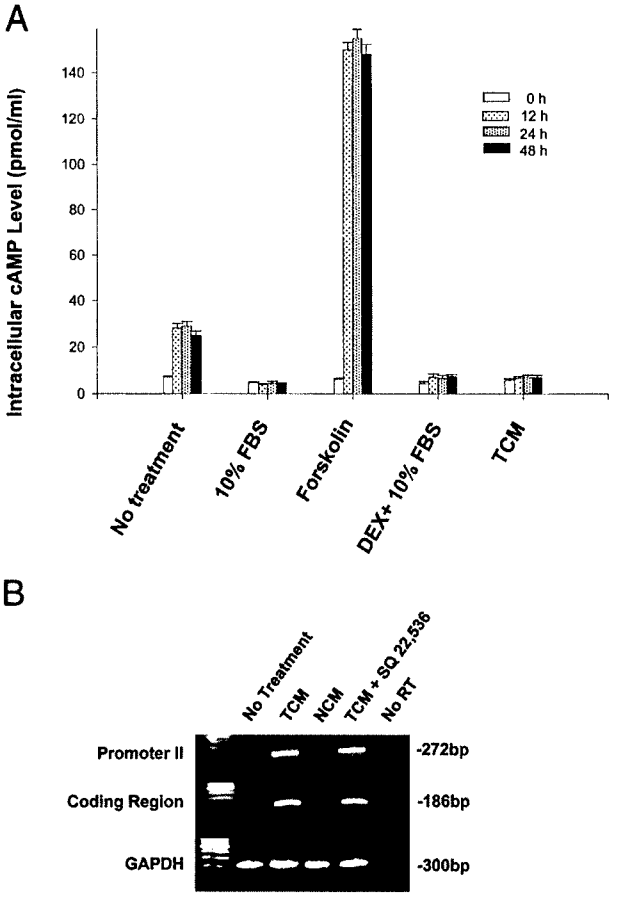

Fig. 2. *A* and *B*, activation of P450arom promoter II by TCM is not cAMP dependent. HAFs were incubated under various conditions [*No treatment*, serum-free DMEM/F12; *10% FBS*, DMEM/F12 + 10% FBS; *Forskolin*, 10 μM forskolin; *DEX+ 10% FBS*, 250 nM DEX + 10% FBS; *TCM*; *NCM*; *TCM + SQ22,536*, TCM + 100 μM SQ 22,536; *No RT*, no reverse transcriptase reaction mixture for negative control]. HAFs were then sampled at 0, 12, 24, and 48 h for intracellular cAMP assay or at 48 h for semiquantitative RT-PCR. *A*, TCM did not increase the intracellular cAMP levels at 12, 24, and 48 h as in treatments with FBS and DEX-FBS. On the other hand, forskolin, an adenylate cyclase inducer, strikingly increased cAMP levels, as expected. *Bars*, SE. *B*, SQ 22,536, an adenylate cyclase inhibitor, could not eliminate the TCM-induced induction of P450arom promoter II-specific transcripts.

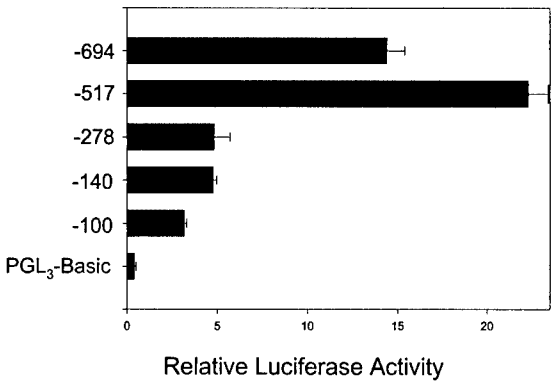

Fig. 3. Regulation of P450arom promoter II activity in primary HAFs. Luciferase plasmids containing the 5′-flanking region of human P450arom promoter II with serial deletions (−100, −140, −214, −278, −517, and −694 bp) were transfected into HAFs. pCMV *Renilla* was used as an internal control for transfection efficiency. Promoter II activity was normalized to pCMV *Renilla* and was represented as the average of data from triplicate replicates; *bars*, SE. The empty luciferase vector PGL3-Basic was arbitrarily assigned a unit of 1, and the rest of the results were expressed as multiples of the PGL3-Basic vector. We conclude that the −517/−278-bp region contains critical stimulatory elements, which regulate the baseline activity.

[4] Internet address: http://www.blast.genome.ad.jp/sit/TFSEARCH.

-550 CATGGTACAA GAGATTTTAG ATCTTCATTG AAGTCACTAG AGATGGCCTG

-500 AGTGAGTCAC TTTGAATTCA ATAGACAAAC TGATGGAAGG CTCTGAGAAG

-450 ACCTCAACGA TGCCCAAGAA ATGTGTTCTT ACTGTAGAAA CTTACTATTT

-400 TGATCAAAAA AGTCATTTTG GTCAAAAAGG GGAGTTGGGA GATTGCCTTT

C/EBP 1 89% C/EBP 2 98%

-350 TTGTTTTGAA ATTGATTTGG CTTCAAGGGA AGAAGATTGC CTAAACAAAA

SF-1 85 %

-300 CCTGCTGATG AAGTCACAAA ATGACTCCAC CTCTGGAATG AGCTTTATTT

CRE 86%

-250 TCTTATAATT TGGCAAGAAA TTTGGCTTTC AATTGGGAAT GCACGTCACT

-200 CTACCCACTC AAGGGCAAGA TGATAAGGTT CTATCAGACC AAGCGTCTAA

SF-1 86%

-150 AGGAACCTGA GACTCTACCA AGGTCAGAAA TGCTGCAATT CAAGCCAAAA

-100 GATCTTTCTT GGGCTTCCTT GTTTTGACTT GTAACCATAA ATTAGTCTTG

TATA

-50 CCTAAATGTC TGATCACATT ATAAAACAGT AAGTGAATCT GTACTGTACA

Fig. 4. Potential *cis*-acting elements located in the P450arom promoter II region. A computer-assisted search revealed two C/EBP binding sites at −350/−337 bp and −317/−304 bp, located within the −517/−278 bp region of promoter II. Additionally, two previously identified SF-1 sites and a CRE are present within the −278/−100-bp region. The percentages depict the homology to the consensus sequences.

site completely abolished both baseline and TCM-induced activities. Mutation of the two SF-1 sites or the −350/−337-bp C/EBP binding site did not effect TCM induction of promoter II activity (Fig. 5). We found that NCM did not change the activity of the −517-bp construct in comparison with incubation with DMEM/F12 only (data not shown). Therefore, promoter II activity in NCM-treated HAFs is similar to the baseline level. In this particular experiment illustrated in Fig. 5, we determined the TCM fold induction of promoter in comparison with NCM treatment.

Induction of Promoter II Activity in HAFs by Factors Derived from T47D Breast Cancer Cells Is Mediated by C/EBPβ. Fig. 6 depicts the effects of the adipogenic transcription factors, C/EBPα, C/EBPβ, and C/EBPδ, on the activity of the −517-bp promoter II construct in HAFs. Ectopic expressions of C/EBPβ (3.5-fold) and C/EBPα (2.5-fold) stimulated promoter II activity, whereas C/EBPδ did not have any significant effect (Fig. 6).

Thus far, these results were indicative of TCM induction of promoter II activity via a C/EBP binding site (−317/−304 bp). Ectopic expressions of C/EBPα and C/EBPβ significantly stimulated the −517-bp promoter II construct (Fig. 6). To determine whether C/EBPα or C/EBPβ mediates TCM induction of promoter II, EMSA was used using an oligonucleotide probe (−322/−303 bp) containing the −317/−304-bp C/EBP binding site, nuclear extracts from HAFs incubated with or without TCM, and supershifting antibodies against C/EBPα, C/EBPβ, and C/EBPδ. This C/EBP binding site (−317/−304 bp) was chosen to be included in the probe, because this element was found to be critical for TCM activation of promoter II (Fig. 5). We identified two specific complexes (1 and 2) as verified by wild-type and mutated cold competitors in TCM-treated HAFs (Fig. 7). Antibodies against both C/EBPβ and C/EBPδ supershifted complex 1, indicating the presence of C/EBPβ and C/EBPδ. On the other hand, antibodies against C/EBPα or CREB did not eliminate or supershift any of these complexes. To further investigate whether the activation of P450arom promoter II is mediated by C/EBPβ, we demonstrated that the effects of TCM and C/EBPβ were not additive. TCM stimulated the −517 construct by 6-fold, whereas the addition of C/EBPβ to TCM did not further increase this induction, which was suggestive that the effects of TCM on promoter II were, at least in part, mediated by C/EBPβ (Fig. 7*B*).

These experiments were suggestive that TCM induction of promoter II activity was mediated by C/EBPβ but not by C/EBPα or C/EBPδ, because C/EBPα does not bind to the regulatory element at −317/−304 bp, which is critical for TCM stimulation of promoter II. Although C/EBPδ binds to this site, ectopic expression of C/EBPδ does not increase promoter II activity. To confirm this conclusion, we determined the effects of TCM on the mRNA levels of C/EBP isoforms in HAFs. Treatments with TCM or NCM did not change the mRNA levels of C/EBPα or C/EBPδ. On the other hand, only TCM induced C/EBPβ expression in HAFs strikingly (Fig. 8). Thus, we

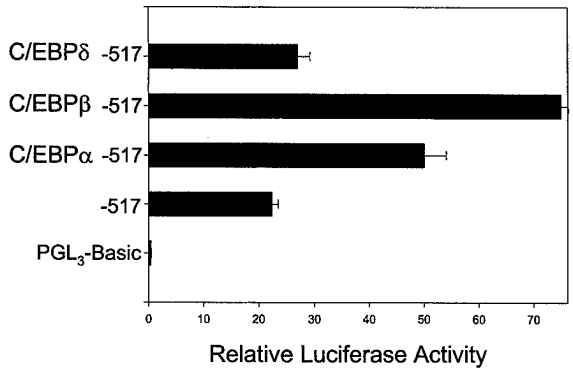

Fig. 6. The effects of adipogenic transcription factors, C/EBPα, C/EBPβ, and C/EBPδ, on the activity of the −517-bp promoter II construct in HAFs. Mammalian expression vectors of C/EBPs were cotransfected into HAFs, together with the −517-bp promoter II construct. Ectopic expressions of C/EBPβ (3.5-fold) and C/EBPα (2.5-fold) stimulated promoter II activity, whereas C/EBPδ did not have any significant effects. *Bars,* SE.

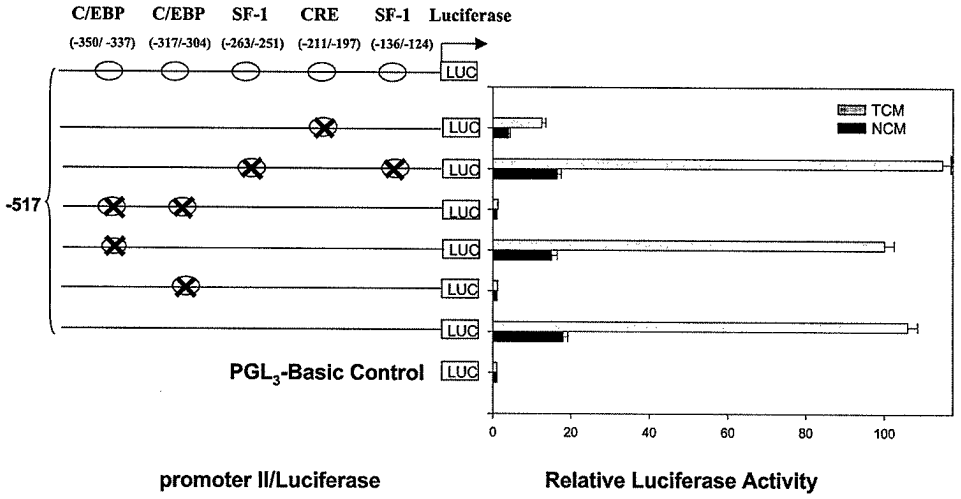

Fig. 5. A C/EBP binding site (−317/−304 bp) is essential for both basal and TCM-induced activation of promoter II. TCM induced the activity of the −517-bp construct by 5.7-fold as compared with NCM treatment. Site-directed mutagenesis of five potentially important *cis*-acting elements demonstrated that a CRE (−211/−197 bp) and a C/EBP binding site (−317/−304 bp) were essential for TCM induction of promoter II activity. In particular, mutation of the −317/−304-bp C/EBP binding site completely abolished both baseline and TCM-induced activities. Mutations of the two SF-1 sites or another C/EBP binding site (−350/−337 bp) did not affect TCM induction of promoter II activity. *Bars,* SE.

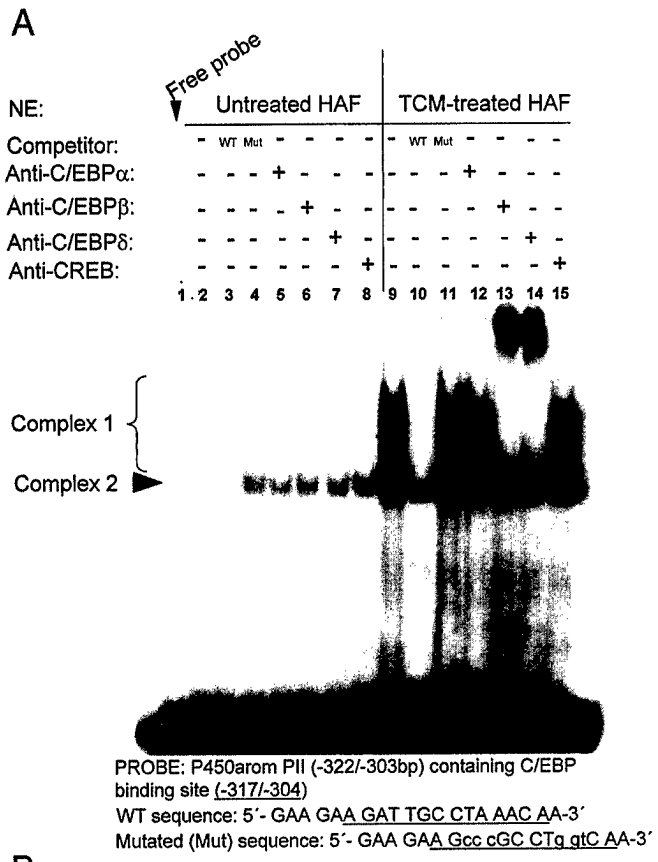

Fig. 7. The effects of TCM on promoter II is mediated by C/EBPβ. *A*, C/EBPβ and C/EBPδ bind to the C/EBP site (−317/−304 bp) upstream of promoter II in TCM-treated HAFs. EMSA was used with an oligonucleotide probe (−322/−303 bp) containing the −317/−304-bp C/EBP binding sequence, nuclear extracts from HAFs incubated with or without TCM, and supershifting antibodies against C/EBPα, C/EBPβ, C/EBPδ, and CREB. We identified two specific complexes (1 and 2) as verified by wild-type (*WT*) and mutated (*Mut*) cold competitors in TCM-treated HAFs. Antibodies against C/EBPβ or C/EBPδ supershifted complex 1, indicating the presence of C/EBPβ and C/EBPδ in this complex. On the other hand, C/EBPα or CREB did not eliminate or supershift any of these complexes. *B*, TCM induced the activity of the −517-bp promoter II construct by six-fold, whereas the addition of C/EBPβ to TCM did not increase the promoter II activity any further. *Bars*, SE.

conclude that TCM induction of P450arom promoter II in HAF is mediated, at least in part, by the induction of the expression of C/EBPβ, which binds to the −317/−304 bp region in this promoter.

DISCUSSION

The understanding of the molecular mechanisms that are responsible for aberrant P450arom expression in tumor-bearing breast adipose tissue may provide insights into the etiology of breast cancer and lead to the identification of molecular targets for the development of novel treatment strategies. Investigators from at least four different laboratories have demonstrated strikingly increased levels of aromatase activity and P450arom mRNA in breast adipose tissue containing a tumor compared with breast tissue from disease-free women (6, 7, 14–16). It was also consistently found that up-regulation of promoter II activity was responsible, in part, for increased aromatase expression in breast cancer (14–16). Although it was suggested that promoter II up-regulation by breast tumors might be mediated by prostaglandin E_2 and cAMP, no direct evidence to support this concept has been provided to date (26). Another report, on the other hand, supports our findings regarding the effect of MCF-7 breast cancer cells on switching the promoter use from I.4 to II (27). The downstream signal transduction events or the specificity of breast epithelial cell types, however, has not been characterized in this article (27). We herein present data to support that malignant breast cells induce aromatase expression via promoter II using a cAMP-independent mechanism. A key event is the binding of the adipogenic factor C/EBPβ to a specific *cis*-acting element upstream of promoter II to activate its transcription. We have used an *in vitro* system to support our conclusion. The use of malignant and normal breast epithelial cell conditioned media with clear and consistent biological effects on fibroblasts and the use of positive and negative controls for cell types and various components of signal transduction pathways, however, offset the disadvantages of using an *in vitro* system and permit the performance of useful mechanistic experiments. Additionally, our conclusions are supported by *in vivo* data from human breast cancer specimens, which showed down-regulation of C/EBPα but persistent expression of C/EBPβ and C/EBPδ proximal to malignant cells (20). On the basis of these data, we suggest the following model. Breast cancer cells secrete cytokines that selectively down-regulate essential adipogenic factors, which inhibit the differentiation of fibroblasts to mature adipocytes. Estrogen production in these fibroblasts maintained in the undifferentiated state by malignant cells is further enhanced by tumor-derived factors, which exist in the T47D or MCF-7 cell conditioned media. These factors act via a cAMP-independent pathway to increase C/EBPβ expression in adipose fibroblasts and enhance the binding of C/EBPβ to a specific promoter II regulatory sequence. The end result is increased local estrogen concentration in the breast tumor.

We do not know yet the identities of the unknown factors in TCM that increase C/EBPβ expression and P450arom promoter II activity in adipose fibroblasts. Cytokines such as TNF-α and IL-6 were shown to increase the transcriptional activity of C/EBPβ (28, 29). It is not clear, however, whether these cytokines increase C/EBPβ expression

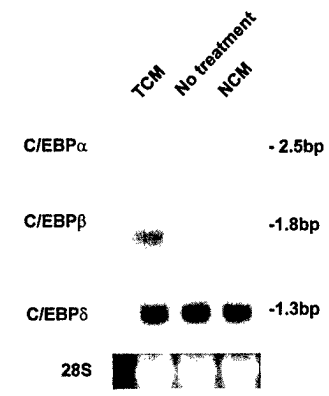

Fig. 8. C/EBPβ transcripts are induced by TCM in HAFs. HAFs were treated by NCM or TCM, or left untreated (*No treatment*) for 48 h. Twenty μg of total RNA isolated from each sample were then used for Northern blot analysis. The 28S RNA fraction was included to demonstrate the presence of comparable amounts of total RNA in each lane. TCM profoundly increased the expression of C/EBPβ, whereas the expression patterns of C/EBPα and C/EBPδ were not altered by TCM treatment.

or promoter II activity. Our preliminary findings and previous publications demonstrated that these cytokines (IL-11, IL-6, and TNFα) do not activate P450arom promoter II, which is up-regulated *in vivo* in breast tumors. Instead, these substances activate promoter I.4, which is not up-regulated in tumors (17, 30–32).[5] Therefore, these cytokines by themselves probably do not account for the *in vivo* up-regulation of aromatase expression in breast tumors. Our efforts will continue to identify these unknown factors originating from malignant cells to induce aromatase expression in the adipose fibroblast via promoter II.

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REFERENCES

1. McNatty, K. P., Baird, D. T., Bolton, A., Chambers, P., Corker, C. S., and Maclean, H. Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle. J. Endocrinol., *77:* 77–85, 1976.
2. Ackerman, G. E., Smith, M. E., Mendelson, C. R., MacDonald, P. C., and Simpson, E. R. Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. J. Clin. Endocrinol. Metab., *53:* 412–417, 1981.
3. Price, T., Aitken, J., Head, J., Mahendroo, M., Means, G. D., and Simpson, E. R. Determination of aromatase cytochrome P450 messenger RNA in human breast tissues by competitive polymerase chain reaction (PCR) amplification. J. Clin. Endocrinol. Metab., *74:* 1247–1252, 1992.
4. Hemsell, D. L., Grodin, J. M., Brenner, P. F., Siiteri, P. K., and MacDonald, P. C. Plasma precursors of estrogen. J. Clin. Endocrinol. Metab., *38:* 476–479, 1974.
5. James, V. H., Reed, M. J., Lai, L. C., Ghilchik, M. W., Tait, G. H., Newton, T. J., and Goldham, N. G. Regulation of estrogen concentrations in human breast tissues. Ann. NY Acad. Sci., *595:* 227–235, 1990.
6. O'Neill, J. S., Elton, R. A., and Miller, W. R. Aromatase activity in adipose tissue from breast quadrants: a link with tumor site. Br. Med. J., *296:* 741–743, 1988.
7. Bulun, S. E., Price, T. M., Mahendroo, M. S., Aitken, J., and Simpson, E. R. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcriptions using competitive polymerase chain reaction after reverse transcription. J. Clin. Endocrinol. Metab., *77:* 1622–1628, 1993.
8. Yue, W., Wang, J. P., Hamilton, C. J., Demers, L. M., and Santen, R. J. *In situ* aromatizaton enhances breast tumor estradiol levels and cellular proliferation. Cancer Res., *58:* 927–932, 1998.
9. Brodie, A. Aromatase and its inhibitors–an overview. J. Steroid Biochem. Mol. Biol., *40:* 255–261, 1991.
10. Santen, R. J. Clinical use of aromatase inhibitors in human breast carcinoma. J. Steroid Biochem. Mol. Biol., *40:* 247–253, 1991.
11. Santen, R. J. Estrogen synthesis inhibitors from "off the rack" to haute couture. J. Clin. Endocrinol. Metab., *77:* 316–318, 1993.
12. Mahendroo, M. S., Mendelson, C. R., and Simpson, E. R. Tissue-specific and hormonally controlled alternative promoters regulate aromatase *cytochrome P450* gene expression in human adipose tissue. J. Biol. Chem., *268:* 19463–19470, 1993.
13. Zhao, Y., Mendelson, C. R., and Simpson, E. R. Characterization of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes. Mol. Endocrinol., *9:* 340–349, 1995.
14. Agarwal, V. R., Bulun, S. E., Leitch, M., Rohrich, R., and Simpson, E. R. Use of alternative promoters to express the aromatase cytochrome P450 (*CYP19*) gene in breast adipose tissues of cancer-free and breast cancer patients. J. Clin. Endocrinol. Metab., *81:* 3843–3849, 1996.
15. Zhou, C., Zhou, D., Esteban, J., Murai, J., Siiteri, P. K., Wilczynski, S., and Chen, S. Aromatase gene expression and its exon I usage in human breast tumors. J. Steroid Biochem. Mol. Biol., *59:* 163–171, 1996.
16. Harada, N. Aberrant expression of aromatase in breast cancer tissues. J. Steroid Biochem. Mol. Biol., *61:* 175–184, 1997.
17. Nichols, J. E., Bulun, S. E., and Simpson, E. R. Effects of conditioned medium from different cultured cell types on aromatase expression in adipose stromal cells. J. Soc. Gynecol. Investig., *2:* 45–49, 1995.
18. Singh, A., Purohit, A., Coladham, N. G., Ghilchik, M. W., and Reed, M. J. Biochemical control of breast aromatase. Breast Cancer Res. Treat., *49* (Suppl. 1)*:* S9–S14; discussion, S33–S37, 1998.
19. Miller, W. R., Mullen, P., Sourdaine, P., Watson, C., Dixon, J. M., and Telford, J. Regulation of aromatase activity within the breast. J. Steroid Biochem. Mol. Biol., *61:* 193–202, 1997.
20. Meng, L., Zhou, J. F., Sasano, H., Suzuki, T., Zeitoun, K. M., and Bulun, S. E. Tumor necrosis factor α and interleukin-11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively downregulating C/EBPα and PPARγ: mechanism of desmoplastic reaction. Cancer Res., *61:* 2250–2255, 2001.
21. Hu, E., Tontonoz, P., and Spiegelman, B. M. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc. Natl. Acad. Sci. USA, *92:* 9856–9860, 1995.
22. Zhang, B., Berger, J., Hu, E., Szalkowski, D., White-Carrington, S., Spiegelman, B. M., and Moller, D. E. Negative regulation of peroxisome proliferator-activated receptor-γ gene expression contributes to the antiadipogenic effects of tumor necrosis factor-α. Mol. Endocrinol., *10:* 1457–1466, 1996.
23. Cao, Z., Umek, R. M., and McKnight, S. L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3–L1 cells. Genes Dev., *5:* 1538–1552, 1991.
24. Zeitoun, K., Takayama, K., Michael, M. D., and Bulun, S. E. Stimulation of aromatase P450 promoter II activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same *cis*-acting element. Mol. Endocrinol., *13:* 239–253, 1999.
25. Michael, M. D., Michael, L. F., and Simpson, E. R. A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of human aromatase P450 (*CYP 19*) gene. Mol. Cell. Endocrinol., *134:* 147–156, 1997.
26. Zhao, Y., Agarwal, V. R., Mendelson, C. R., and Simpson, E. R. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE_2 via cyclic AMP, leading to activation of promoter II of the *CYP 19* (aromatase gene). Endocrinology, *137:* 5739–5742, 1996.
27. Harada, N., and Honda, S. Molecular analysis of aberrant expression of aromatase in breast cancer tissues. Breast Cancer Res. Treat., *49* (Suppl. 1)*:* S15–S21; discussion S33–S37, 1998.
28. Baer, M., Williams, S. C., Dillner, A., Schwartz, R. C., and Johnson, P. F. Autocrine signals control CCAAT/enhancer binding protein β expression, localization, and activity in macrophages. Blood, *92:* 4353–4365, 1998.
29. Yin, M., Yang, S. Q., Lin, H. Z., Lane, M. D., Chatterjee, S., and Diehl, A. M. Tumor necrosis factor α promotes nuclear localization of cytokine-inducible CCAAT/enhancer binding protein isoforms in hepatocytes. J. Biol. Chem., *271:* 17974–17978, 1996.
30. Crichton, M. B., Nichols, J. E., Zhao, Y., Bulun, S. E., and Simpson, E. R. Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. Mol. Cell. Endocrinol., *118:* 215–220, 1996.
31. Singh, A., Purohit, A., Duncan, L. J., Mokbel, K., Ghilchik, M. W., and Reed, M. J. Control of aromatase activity in breast tumors: the role of the immune system. J. Steroid Biochem. Mol. Biol., *61:* 185–192, 1997.
32. Zhao, Y., Nichols, J. E., Valdez, R., Mendelson, C. R., and Simpson, E. R. Tumor necrosis factor-α stimulates aromatase gene expression in human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter I.4. Mol. Endocrinol., *10:* 1350–1357, 1996.

[5] J. Zhou, B. Gurates, S. Yang, S. Sebastian, and S. E. Bulun, unpublished observations.